MASTER OF SCIENCE

The Clinical Determinants of Plasma β-amyloid and Its Association with Structural and Functional Vascular Changes

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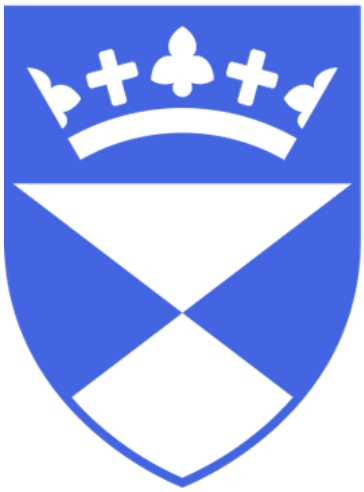

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ABSTRACT

Background: β-amyloid plaque deposition is a pathological hallmark of Alzheimer's disease and cerebral amyloid angiopathy. However, β-amyloid has also been shown to possess direct vasoactive properties and increased plasma β-amyloid has been associated with endothelial dysfunction in mice.

Aims: To investigate the metabolic and pharmacological determinants of plasma β-amyloid levels and to investigate the association of plasma β-amyloid with structural and functional markers of vascular integrity as well as cardiovascular outcomes.

Methods: Plasma β-amyloid 40 and β-amyloid 42 levels were measured in 407 subjects with type 2 diabetes (T2DM) and 245 subjects without T2DM from the Surrogate markers for Micro and Macro vascular hard endpoints for Innovative diabetes Tools (SUMMIT) consortium database. The SUMMIT database was used to analyse factors associated with altered plasma β-amyloid levels and to determine the relationship between plasma β-amyloid and biomarkers of cardiovascular health and disease. Biomarkers analysed included, reactive hyperaemia index (RHI) with EndoPAT, reactive hyperaemia in response to occlusion, arterial stiffness, skin microcirculation response to acetylcholine (ACh) and sodium nitroprusside (SNP) and carotid intima-media thickness (IMT).

Results: In the SUMMIT baseline cohort as well as in T2DM and non-T2DM sub-groups, renal function as estimated by estimated glomerular filtration rate (eGFR) was the most significant independent predictor of plasma β-amyloid 40 and 42 levels. In the T2DM subgroup, insulin use was also found to be independently associated with increased β-amyloid 40 and 42 levels. Use of diuretics was independently associated with increased β-amyloid 40 and 42 levels in the SUMMIT baseline cohort and increased β-amyloid 40 levels in the T2DM cohort. After adjusting for conventional cardiovascular risk factors of age, gender, diabetes status, systolic blood pressure (SBP), high-density lipoprotein (HDL) cholesterol and total cholesterol as well as independent predictors of plasma β-amyloid, β-amyloid 40 was independently associated with increased arterial stiffness as measured by pulse wave velocity, and reduced vascular responsiveness to ACh and SNP in the SUMMIT baseline and SUMMIT T2DM cohorts. β-amyloid was not found to be a significant predictor of cardiovascular outcomes over 4-6 years of follow up.

Conclusions: The research in this thesis shows that plasma β-amyloid levels are affected by a range of metabolic and pharmacological factors. Future studies should therefore take into account the importance of adjusting for factors such as eGFR, diuretic use or insulin use. The results also show that higher levels of β-amyloid 40, and to a lesser extent 42, are associated with increased arterial stiffness as well as impaired vascular responsiveness to endothelium-dependent and independent stimuli.

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Lastly and perhaps most importantly, I am eternally grateful to my friends and family, who have supported me along the way and without whom this endeavour would have never been successful.

Declaration

"I declare that the content of this project report is my own work and has not previously been submitted for any other assessment. The report is written in my own words and conforms to the University of Dundee's Policy on plagiarism and academic dishonesty. Unless otherwise indicated, I have consulted all of the references cited in this report."

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Signed:

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ABPI	Ankle-brachial pressure index	29
ACE	Angiotensin converting enzyme	36
ACh	Acetylcholine	22
AD	Alzheimer's disease	22
ApoE4	Apolipoprotein E4	91
APP	Amyloid Precursor Protein	24
ARB	Angiotensin II receptor blocker	45
ATP	Adenosine tri-phosphate	90
BACE1	Beta site amyloid precursor protein cleaving enzyme 1	24
BMI	Body Mass Index	19
CAA	Cerebral amyloid angiopathy	89
CABG	Coronary artery bypass graft	94
CC	Common carotid	86
CHD	Coronary heart disease	94
cIMT	Carotid intima-media thickness	92
CRP	C-reactive protein	22
CVD	Cardiovascular disease	19
DBP	Diastolic blood pressure	87
ECM	Extra-cellular matrix	141
eGFR	Estimated glomerular filtration rate	31
FDA	Food and Drug Administration	81
FGF-23	Fibroblast growth factor 23	22
GDF-15	Growth Differentiation Factor 15	22
HDL	High Density Lipoprotein	20
ICAM-1	Intercellular adhesion molecule 1	22
IL-1	Interleukin 1	22
IL-6	Interleukin 6	22
IMT	Intima-media thickness	87
LDL	Low density lipoprotein	20
LEAD	Lower extremity arterial disease	95

MMP-1	Matrix metalloproteinase 1	22
MMP-2	Matrix metalloproteinase 2	22
MMP-9	Matrix metalloproteinase 9	22
NO	Nitric Oxide	84
NSAID	Non-steroidal anti-inflammatory drug	27
PCI	Percutaneous coronary intervention	95
RHI	Reactive Hyperaemia Index	83
SBP	Systolic blood pressure	31
SNP	Sodium Nitroprusside	85
T2DM	Type 2 diabetes	26
TIA	Transient Ischaemic Attack	95
TNFα	Tumour necrosis factor alpha	22
VCAM-1	Vascular cell adhesion protein 1	22
VSMC	Vascular smooth muscle cell	85

1 Preface

1.1 The Burden of Cardiovascular Disease

In recent years, non-communicable diseases such as cardiovascular disease (CVD), stroke and diabetes have become the number one killers in the world. In the UK, CVD is responsible for approximately 26% of all deaths and is thus a major public health concern of the nation (1). The global obesity and diabetes epidemic has risen hand in hand with the rise in proportion of deaths caused by CVD. It is now estimated that in the UK, 1 in every 16 people has diabetes, which equates to more than double the rates seen 20 years ago (2). Yet, more concerning statistics suggest that almost a third of all adults in England are now classified as being obese, with a body mass index (BMI) > 30 (2). Due to these prevailing trends, the burden of CVD is expected to rise even further and as such, many nations have made it a priority to tackle this epidemic by means of primary prevention (3). From a therapeutic perspective, several classes of pharmacological agents exist, ranging from lipid lowering therapy, glucose lowering agents or anti-hypertensive drugs. However, given that a significant proportion of the adult population is now likely to have diabetes or obesity, it has become a challenge to identify those people at highest risk of cardiovascular complications. Therefore, there is an increasing need to explore new methods to improve stratification of the population at risk.

1.2 Biomarkers of Cardiovascular Disease

A biomarker is defined as a naturally occurring molecule, gene or characteristic by which a particular pathological or physiological process can be identified (4). Major studies carried out in the 1960s and onwards have provided insight into biomarkers associated with an increased risk of CVD. As a result, the term “risk factor” was coined to describe factors such as hypertension, diabetes, smoking or hypercholesterolaemia (5). These have in turn been combined to create comprehensive risk score calculators, predicting a patient’s risk of developing cardiovascular disease. Over 50 years later, knowledge gained from these studies still hold true and are in widespread use clinically.

The hallmark Framingham Heart Study was the first large-scale prospective cohort study that set out to investigate the epidemiology and aetiology of atherosclerotic and hypertensive CVD (5). The original study cohort consisted of 5,209 adults from Framingham, USA, aged 30-62 years. Additionally, an off-spring cohort and third generation cohort were then studied (6). As such, the Framingham heart study was not only one of the first studies to provide information on the aetiology of CVD, but also provided valuable information about the heritability of these conditions and common comorbidities such as obesity or diabetes. Based on this information, the first method for calculating cardiovascular risk scores, the Framingham Risk Score, was developed (5). Using factors such as age, gender, total cholesterol, high density lipoprotein (HDL) cholesterol, smoking, diabetes and blood pressure, it calculates an individual's 10-year risk of developing CVD. Other risk scores that have since been developed but operate on a similar principle include the locally used ASSIGN risk score, which also takes into account social deprivation (7), or the SCORE (Systemic COronary Risk Evaluation) based on a pooled dataset of 12 European Prospective Cohort Studies (8). Although subtle differences between the various risk scores exist, they predict risk based on the same set of biomarkers.

1.2.1 Limitations of Conventional Biomarkers and Cardiovascular Risk Scores

Plasma lipid levels are cited as one of the major risk factors for the development of CVD and are thus included in all conventional CVD risk scores. However, a number of limitations of conventional biomarkers exist. Despite extensive research, studies have shown that as many as 50% of individuals who develop coronary heart disease have only one risk factor, and in some age groups, up to 35% of individuals have no conventional CVD risk factors (9). A recent study by Sachdeva et al. found that a substantial proportion of patients presenting with acute myocardial infarction across hospitals in the United States had lipid levels within the recommended range at time of presentation. Of the 48,093 patients without prior history of coronary artery disease, other atherosclerotic disease or diabetes, 41.5% had low density lipoprotein (LDL) cholesterol levels <2.6mmol/L (10). In the UK, estimates suggest that over half

of the adult population have elevated cholesterol levels (11). Due to extensive primary prevention campaigns, the majority of patients identified as being at risk of CVD are likely to be taking statins. Indeed, a cross-sectional study of the prevalence of primary prevention statin prescriptions showed that between 2009-2011, estimated prevalence of statin use was as high as 30% in subjects aged 50 years and over (12). It is possible, that widespread use of lipid lowering agents could account for relatively low-normal cholesterol levels among patients with acute coronary syndromes. Understanding the effects of risk factor modifying therapies on the predictive value of established risk scores is therefore extremely important. However, a recent review of CVD risk scores found that none of them accounted for the effects of risk modifying treatment such as statin use. (13) Therefore, while biomarkers such as LDL and HDL may be of use in predicting CVD risk in a statin-naïve population, their predictive value in the general population with widespread statin use is unclear.

As is the case with lipid lowering agents such as statins or fibrates, the use of antihypertensive drugs is widespread in the UK. It is well established that lower blood pressure is associated with a lower cardiovascular risk (14). However, a common but underestimated limitation of blood pressure as a biomarker is the inaccuracy associated with one-off clinic measurements. A study looking at blood pressure measurements in the primary care setting found that based on poor technique, 24-32% of patients were being misdiagnosed as having systolic hypertension and 15-21% were being misdiagnosed as having diastolic hypertension (15). Consequently, the research into new, easily measurable biomarkers has continued and yielded results with varying levels of success.

1.3 Novel Circulating Biomarkers

Over the last few years, many potential novel biomarkers of CVD risk have been investigated. Broadly, these can be grouped into numerous categories including inflammatory biomarkers, metabolic biomarkers as well as biomarkers of vascular remodelling. While it is beyond the scope of this thesis to discuss all of these

promising novel circulating biomarkers, the following table aims to summarise some of those most commonly discussed in the literature.

Table 1-1: A summary of potential novel circulating biomarkers of CVD risk, table adapted from (16)*, for abbreviations see list of abbreviations.*

Inflammatory	**Metabolic**
CRP (17) TNF-alpha (18,19) IL-1/IL-1 receptor antagonist (20) IL-6 (21) GDF-15 (22)	Adiponectin (23) Leptin (24) FGF-23 (25) Homocysteine (26)
Biomarkers of vascular remodelling	**Biomarkers of endothelial dysfunction**
MMP-1 (27) MMP-2 (28) MMP-9 (29)	I-CAM (30) V-CAM (30) E-selectin (30)

1.4 β-amyloid as a Potential Novel Circulating Biomarker

Another potential, but to date relatively unexplored biomarker of CVD risk is β-amyloid, a peptide thought to be the hallmark of Alzheimer's disease (AD). While an association between AD, β-amyloid and CVD dates far back, only more recently has this association been looked at in further detail. A number of in vitro studies have suggested that β-amyloid may possess direct vasoactive properties (31,32). However, as a general rule two main limitations of previous studies exist. Firstly, the doses of β-amyloid used in studies has often exceeded concentrations seen physiologically in organisms. Secondly, β-amyloid peptides are either used freshly or allowed to form oligomers and subsequently applied acutely. Neither of these scenarios replicate adequately what occurs in vivo, whereby vascular exposure to plasma β-amyloid is a of a chronic nature and at much lower concentrations. Perhaps most important for this study are the currently unpublished findings by Meakin et al. In this animal study, the effect of β-amyloid on endothelial function was investigated. Mice with chronic exogenous β-amyloid 42 infusions were found to have significantly diminished responses to the vasodilator acetylcholine (ACh) as assessed by means of laser Doppler imaging with iontophoresis of vasoactive

chemicals in the skin microcirculation. Additionally, by lowering the levels of circulating β-amyloid using pharmacological inhibition of an enzyme involved in the rate-limiting step of β-amyloid production, the endothelial response to ACh improved significantly (33). Given these findings, the next step was to determine whether findings in animal models translate into the human population. Conveniently, the pre-existing SUMMIT database contained data on plasma β-amyloid levels as well as laser Doppler imaging and iontophoresis assessments and other surrogate structural and functional biomarkers of vascular changes. The purpose of this thesis was therefore to build on previous findings of the research group and, using statistical analysis of data from pre-existing databases, determine whether plasma β-amyloid could serve as a biomarker of CVD risk. More specifically, the aim was to determine whether in humans, similar associations between plasma beta-amyloid and skin microcirculation function exist, whether any associations exist between plasma β-amyloid and other surrogate markers of vascular structure and function and whether plasma β-amyloid levels are associated with adverse cardiovascular outcomes. However, due to the very limited knowledge of the physiological and pathophysiological properties of plasma β-amyloid out with the context of AD, the first step was to establish any clinical determinants of plasma β-amyloid levels.

2 An exploration of the biochemical, metabolic and pharmacological factors affecting plasma β-amyloid levels in the systemic circulation

2.1 Introduction

2.1.1 β Amyloid Production

Aβ is a ~4kDa peptide extensively researched primarily in the context of Alzheimer's disease (AD) (34) (35). In this pathological state, intracerebral accumulation of plaques consisting of β-amyloid represent a pathological hallmark. While the role of β-amyloid in the development of AD is well established, its role in both other disease processes and physiological processes is currently unknown. Present in high concentrations in the brain, β-amyloid is also found in a number of other human tissues including skeletal muscle, liver and kidney (36). In recent years, several studies have suggested that plasma β-amyloid is associated with CVD and may even directly contribute to the pathophysiological process (37–39). However, before the potential predictive value of plasma β-amyloid in establishing CVD risk is determined, more information is required about factors affecting plasma β-amyloid concentrations. The following review will therefore aim to summarise steps in β-amyloid production, as well as our current knowledge of clinical determinants of plasma β-amyloid.

β-amyloid is formed by the sequential processing of its precursor molecule, amyloid precursor protein (APP) (40). APP can be processed down either a non-amyloidogenic pathway, which predominates in health, but also down an amyloidogenic pathway yielding Aβ peptides. The latter is thought to predominate in pathological conditions such as Alzheimer's disease. The first and rate-limiting step in the amyloidogenic pathway is cleavage of APP by β-secretase (BACE1) (Figure 2.1 image from (41). This results in the formation of a membrane bound fragment C99 and a secreted fragment. C99 is further cleaved by gamma-secretase, however, gamma-secretase has a non-specific cleavage site resulting in the formation of a

range of different peptides with varying amino acid lengths. Among the different Aβ peptides, Aβ40 is the most abundant form, while Aβ42 is the main pathological form implicated in AD (42).

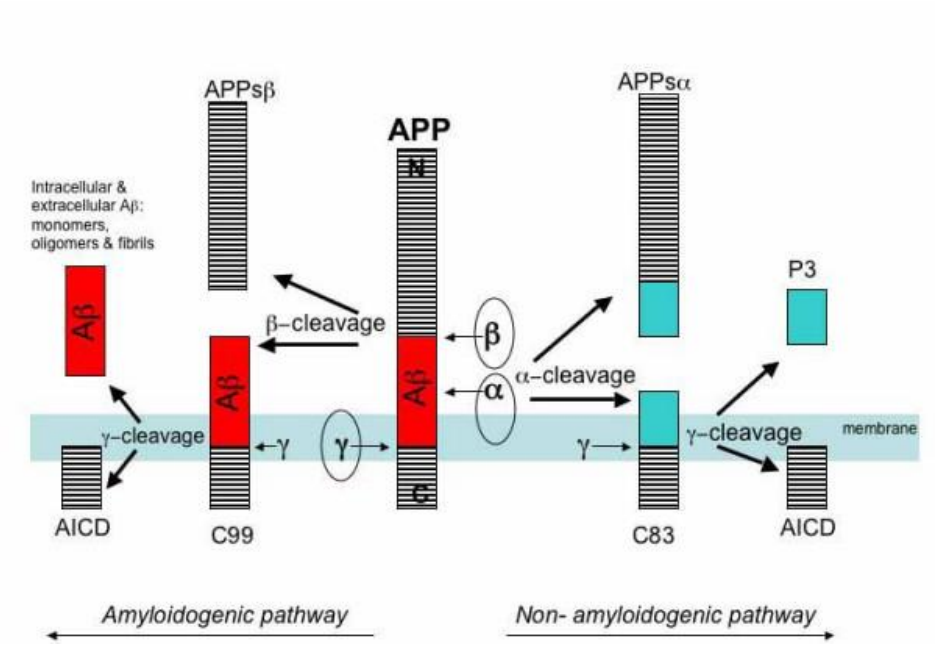

Figure 2-1: Fate of amyloid precursor protein when processed down different pathways. BACE1 mediates beta-cleavage (41).

While the processes leading to β-amyloid production have been well mapped out, β-amyloid degradation or excretion is less understood. A number of different proteases have been implicated in the degradation of β-amyloid peptides. These include metalloproteases such as neprilysin, endothelin converting enzymes, angiotensin converting enzyme, insulin degrading enzyme as well as matrix metalloproteinases 2 and 9 (43) (44–47). Interestingly, all of these proteases have been extensively linked to a number metabolic and CVD processes.

2.1.2 Known Clinical Determinants of β-amyloid Peptides

Although β-amyloid has been investigated primarily in the brain, the discovery of methods enabling the measurement of circulating peripheral β-amyloid have unveiled associations with several different clinical determinants.

A number of studies emerged in the 1990s that, for the first time, depicted an epidemiological association between AD and type 2 diabetes mellitus (T2DM). The Hisiyama study in 1995 reported a relative risk (RR) of 2.18 for AD in patients with type 2 diabetes (48). A study published some years later from the same group made the interesting observation that the RR of AD was greater than that of vascular dementia in patients with diabetes (RR 2.05 vs RR 1.82) (49). These observations prompted further research into the link between the pathogenesis of AD and that of diabetes. Since then, several studies have shown that not only do pathological processes in patients with diabetes predispose individuals to AD, there is also evidence showing that this is a bi-directional relationship. Indeed, several studies discussed below have shown that Aβ affects processes relevant to diabetes such as glucose handling and energy homeostasis. Several independent research groups have found that non-diabetic patients with AD have evidence of impaired glucose tolerance (50). This would suggest that there is perhaps a link between β-amyloid and some earlier stages of type 2 diabetes. Further reinforcing these findings, a study examining 101 pre-school children and 309 adolescent children found that obese adolescent children had significantly higher levels of circulating Aβ42 when compared to normal-weight peers but no significant difference in Aβ42 was observed between obese and normal-weight pre-school children. They found a significant positive correlation between plasma Aβ42 levels and BMI as well as estimated insulin resistance (HOMA-IR) (51). Another study reported that plasma β-amyloid levels were found to increase after glucose loading in patients with AD (52). This is assumed to occur due to a rise in insulin secretion, as other studies have shown that plasma β-amyloid levels increase following exogenous insulin administration and that plasma β-amyloid levels positively correlate with plasma insulin levels (53). Additionally, β-amyloid is found in high concentrations in the pancreas. While the reason behind this observation remains unclear, post mortem analysis of pancreas tissue from 21 patients with type 2 diabetes revealed accumulation of Aβ aggregates within the Islets of Langerhans (54).

A number of other clinical determinants of plasma β-amyloid have also been reported. Several studies have consistently reported an association between increasing age and higher levels of plasma β-amyloid levels (55). Findings of

significant associations between plasma β-amyloid and creatinine levels have also been consistent in the literature (56). A study of 997 older adults showed that African-American race was associated with lower levels of both plasma β-amyloid 40 and 42. Additionally this study also reported a significant association between female gender and lower plasma β-amyloid 42 levels (57). However, the majority of these studies were done in the context of AD research and as such, primarily enrolled elderly individuals.

2.1.3 Effect of Pharmacological Agents on Plasma β-amyloid

In an attempt to investigate the possibility of using plasma β-amyloid concentrations as a biomarker of CVD or AD, a number of studies had set out to determine the effects of medications on plasma levels. One prospective cohort based study followed up 487 subjects with plasma Aβ42 levels measured at baseline and 2.5 years' follow up. The study found a significant association between use of insulin and increased Aβ42 levels at follow up. The herbal supplement gingko biloba as well as fibrates were associated with reduced plasma Aβ42 levels at follow up. There was no association with statins, or non-steroidal anti-inflammatory medications (NSAIDs) and plasma β-amyloid 40 levels were not investigated in this study (58). Another cross sectional study looked at 371 patients with different forms of cognitive impairment. They found no association with plasma β-amyloid 42 levels and cholinesterase inhibitors, vitamin E, statins, NSAIDs or oestrogens. As with the previous study, plasma β-amyloid 40 levels were not measured (55). In rats, a study looking at the effect of various antidepressant medications on a β-amyloid induced depression-like state found that administration of fluoxetine was associated with reduced soluble plasma β-amyloid levels. No association was found with other selective serotonin reuptake inhibitors used in this study. Additionally, this study did not differentiate between plasma β-amyloid 40 and 42 levels (59).

2.2 Aim

A large number of circulating molecules have in the past been investigated as potential biomarkers of CVD risk. However, very few findings have translated into routine clinical practice. While circulating biomarkers may be shown to predict CVD or outcomes, a lack of knowledge about factors affecting their concentrations are often cited as major limiting factors. Therefore before investigating β-amyloid as a potential new CVD risk biomarker, the following analysis will aim to determine what demographic, metabolic and pharmacological factors affect plasma β-amyloid concentrations.

2.3 Hypothesis

The hypothesis is that there is a significant association between circulating β-amyloid levels and a wide range of demographic, metabolic and pharmacological factors.

2.4 Methods

2.4.1 Study Populations

The Surrogate markers for Micro and Macro vascular hard endpoints for Innovative diabetes Tools (SUMMIT) database was used for the purpose of this study. The original SUMMIT cohort consisted of subjects recruited from existing population cohorts and hospital registers at the university hospitals in Malmö (Sweden), Pisa (Italy), Dundee and Exeter (UK) between December 2010 and April 2013 (60,61). For the purpose of this study, only subjects recruited in Exeter and Dundee were used due to availability of plasma β-amyloid and skin microvascular measurements. Therefore, in this study the term SUMMIT cohort refers to a total of 652 subjects recruited at centres in Dundee and Exeter between December 2010 and April 2013, with health outcome follow up until April 2017. The baseline cohort was divided into 4 subgroups depending on type 2 diabetes and CVD status:

1. Patients with diabetes and clinically manifest CVD (n=189),
2. Patients with diabetes but without clinically manifest CVD (n=218)
3. Patients without diabetes but with clinically manifest CVD (n=125)
4. Patients without diabetes and without clinically manifest CVD (n=120).

Diabetes was defined as current or previous episodes of hyperglycaemia (fasting plasma glucose >7.0 mmol/l or random plasma glucose >11.1 mmol/l) or by current treatment with metformin, sulphonylureas or other glucose lowering agents. Clinically manifest CVD was defined as a past medical history of acute MI, unstable angina requiring hospitalisation, coronary revascularisation procedures, stroke, transient ischaemic attack confirmed by specialists, peripheral vascular disease defined as ankle-brachial pressure index (ABPI) <0.9 and intermittent claudication or prior angioplasty/ above ankle amputation. Subjects with diabetes were matched across centres for gender, age and duration of diabetes. Subjects without diabetes were matched for gender and age across centres. Exclusion criteria at recruitment were renal replacement therapy, malignancy requiring active treatment, end-stage renal disease, chronic inflammatory disease on therapy, previous bilateral carotid artery invasive interventions or age <40 years (61). Plasma β-amyloid was measured

as a one-off measurement using the Quanterix β-amyloid assay on the Simoa HD-1 analyser (62). This allowed for a more sensitive measure at the lower ranges of β-amyloid concentrations compared to other available platforms. The study was approved by the local ethical review boards and performed in accordance with the principles of the Declaration of Helsinki. All study subjects provided written informed consent.

2.4.2 Statistical Analysis of Clinical Patient Characteristics and Plasma β-amyloid

All statistical analysis was performed using the IBM SPSS software version 25. Due to population size, data distribution was assessed for normality using the Shapiro-Wilk test with Kolmogorov-Smirnov used as a reference. For the analysis of continuous variables univariate correlations were used. Where both variables had a normal distribution, Pearson's correlation was used. Where variables were found to not have a normal distribution, Spearman's rho was used. To adjust for multiple comparisons, the Bonferroni method was used to adjust the level of significance. By dividing the standard level of significance, p=0.05, by the number of comparisons made a new level of significance was established. In order to analyse the association between plasma β-amyloid and binary determinants such as gender, CVD status, T2DM status or medications use, the Mann-Whitney or Independent T-tests were used, depending on the distribution of variables. Based on significant univariate correlations and associations from the above analyses, linear regression was used to determine independent predictors of plasma β-amyloid 40 and 42 levels in the SUMMIT baseline cohort as well as in subgroups divided based by diabetes status.

2.5 Results

2.5.1 SUMMIT Population Descriptive Statistics

As mentioned before, the SUMMIT cohort consists of 4 groups based on diabetes and CVD status. The following table summarises the baseline characteristics of the SUMMIT cohort. Where the variable presented is a count, the number in brackets

represents the equivalent %, where the number presented is a continuous variable, the number in brackets represents the standard deviation of that variable. Where significant differences exist between groups, the significance is illustrated using an asterisk (*= p<0.05, **= p<0.01, *** = p<0.001). SBP= systolic blood pressure, T2D = type 2 diabetes, DBP = diastolic blood pressure, eGFR = estimated glomerular filtration rate, ACR = albumin:creatinine ratio).

Table 2-1: Summary of baseline patient characteristics in the SUMMIT cohort.

SUMMIT Cohort				
	T2D with CVD	T2D no CVD	No T2D no CVD	No T2D with CVD
N	189	218	120	125
Males n (%) * **	142 (75%)	120 (55%)	53 (44%)	94 (75%)
Age (SD) * **	67.7 (8.0)	63.9 (8.7)	63.1 (8.0)	68.5 (7.5)
T2D Duration (years) * **	12.0 (8.3)	8.6 (6.1)	NA	NA
BMI (kg/m2) * **	31.3 (5.1)	32.6 (5.9)	26.8 (4.3)	28.1 (4.1)
Medication				
Statin use * **	168 (89%)	162 (74%)	20 (17%)	108 (86%)
Antihypertensive use * **	169 (89%)	138 (63%)	18 (15%)	93 (74%)
Blood Pressure				
SBP * **	132.5 (18.1)	133.2 (16.3)	130.2 (16.4)	131.5 (18.3)
DBP	73.1 (8.2)	77.3 (8.8)	77.1 (9.0)	74.8 (8.9)
Metabolic parameters				
HbA1c mmol/mol * **	61.9 (15.6)	59.0 (14.8)	40.1 (4.1)	39.0 (3.3)
Total Cholesterol mmol/l * **	3.8 (0.9)	4.1 (0.9)	5.4 (1.0)	4.2 (0.9)
LDL Cholesterol mmol/l * **	1.8 (0.7)	2.0 (0.8)	3.1 (0.9)	2.2 (0.8)
HDL Cholesterol mmol/l * **	1.2 (0.3)	1.3 (0.4)	1.6 (0.4)	1.4 (0.4)
Triglycerides mmol/l * **	1.8 (1.1)	1.8 (1.0)	1.4 (0.8)	1.3 (0.7)
Renal Function				
Serum Creatinine umol/l * **	92.6 (33.2)	78.0 (20.3)	74.3 (13.7)	84.0 (19.4)
ACR mg/mmol * **	6.9 (34.3)	2.7 (5.5)	0.9 (1.1)	1.9 (4.3)
eGFR mL/min/1.73 m² * **	77.2 (22.3)	86.1 (22.6)	86.9 (16.2)	82.0 (17.5)

2.5.2 Association between Plasma β-amyloid 40 and 42

Before investigating the associations between plasma β-amyloid and clinical variables, we examined the association between plasma β-amyloid 40 and 42. A strong positive correlation was observed as seen in Fig. 2.2.

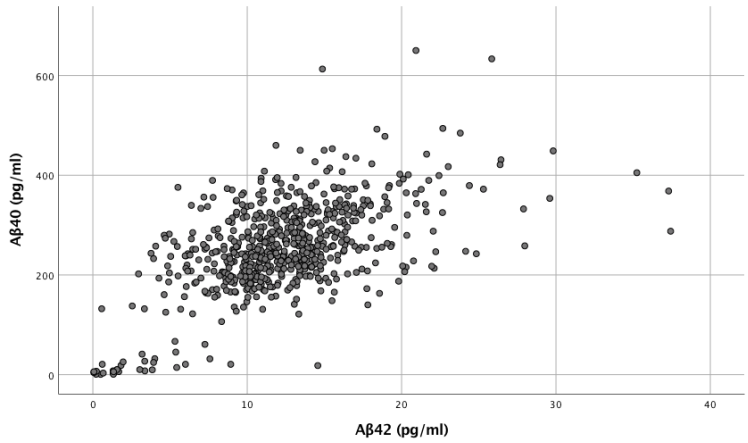

Figure 2-2: Scatterplot of β-amyloid 40 and 42 in n=643 subjects, r=0.494, p=1.1E-40.

2.5.3 Association of Plasma β-amyloid Levels with Continuous Determinants – SUMMIT Cohort

In order to begin exploring the relationship between plasma β-amyloid and baseline continuous variables, univariate correlations were first used looking at the whole cohort, without subdividing subjects into groups.

Table 2-2: Summary of correlations of plasma β-amyloid 40 and 42 and baseline subject characteristics in the SUMMIT cohort. Values shown in bold are significant at Bonferroni adjusted p-value 0.003.

Variable	Correlation	Aβ40 (pg/ml)	Aβ42 (pg/ml)
Age (years)	Correlation Coefficient	0.178	0.14
	Sig.	**7.00E-06**	**4.51E-04**
	N	628	628
Body Mass Index (kg/m2)	Correlation Coefficient	0.109	0.046
	Sig.	0.006	0.253
	N	628	628
Height (m)	Correlation Coefficient	-0.146	-0.104
	Sig.	**1.97E-04**	0.008
	N	642	642
Weight (kg)	Correlation Coefficient	0.024	-0.005

	Sig.	0.544	0.902
	N	642	642
HbA1c (mmol/mol)	Correlation Coefficient	0.099	0.103
	Sig.	0.013	0.009
	N	632	632
Total Cholesterol (mmol/l)	Correlation Coefficient	-0.126	-0.125
	Sig.	**0.002**	**0.002**
	N	632	632
LDL Cholesterol (mmol/l)	Correlation Coefficient	-0.14	-0.179
	Sig.	**0.001**	**1.20E-05**
	N	589	589
HDL Cholesterol (mmol/l)	Correlation Coefficient	-0.102	-0.026
	Sig.	0.011	0.509
	N	626	626
Triglycerides (mmol/l)	Correlation Coefficient	0.072	0.05
	Sig.	0.071	0.213
	N	621	621
eGFR (mL/min/1.73 m²)	Correlation Coefficient	-0.354	-0.367
	Sig.	**1.15E-15**	**8.95E-17**
	N	483	482

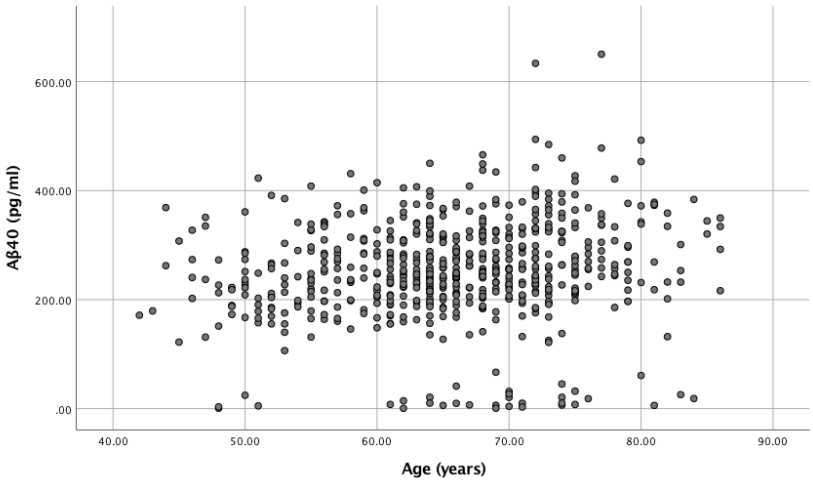

Figure 2-3: Scatter plot of age and β-amyloid 40 in n=628 subjects, r=-0.178, p=7.0E-6.

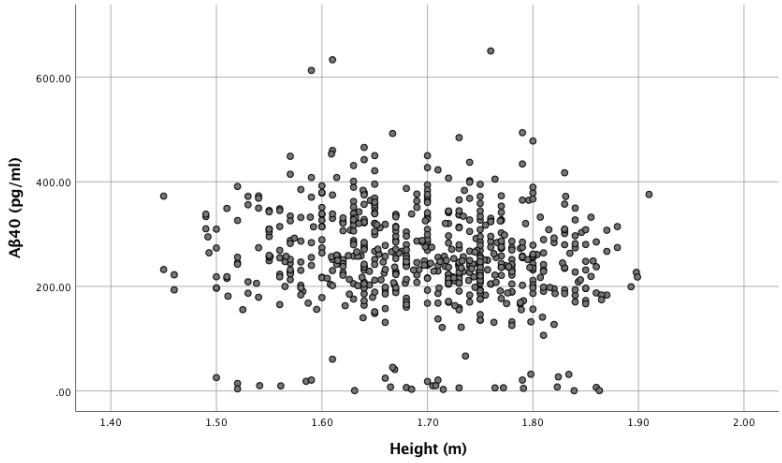

Figure 2-4: Scatter plot of height and β-amyloid 40 in n=642 subjects, r=-0.146, p=1.97E-4.

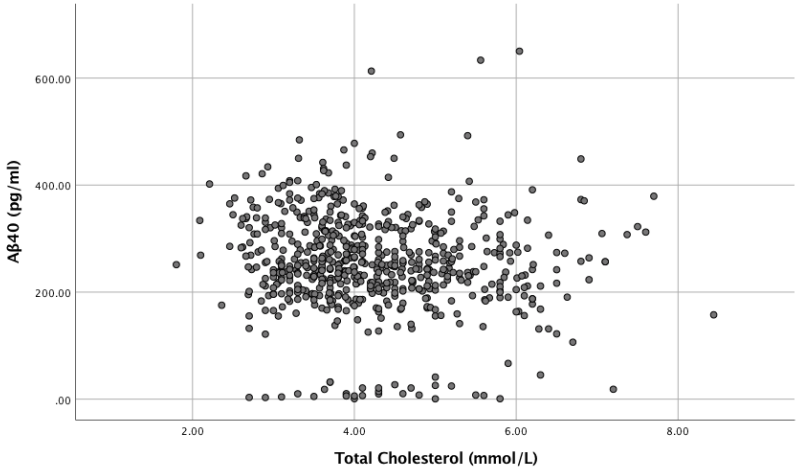

Figure 2-5: Scatter plot of total cholesterol and β-amyloid 40 in n=632 subjects, r=-0.126. p=0.002.

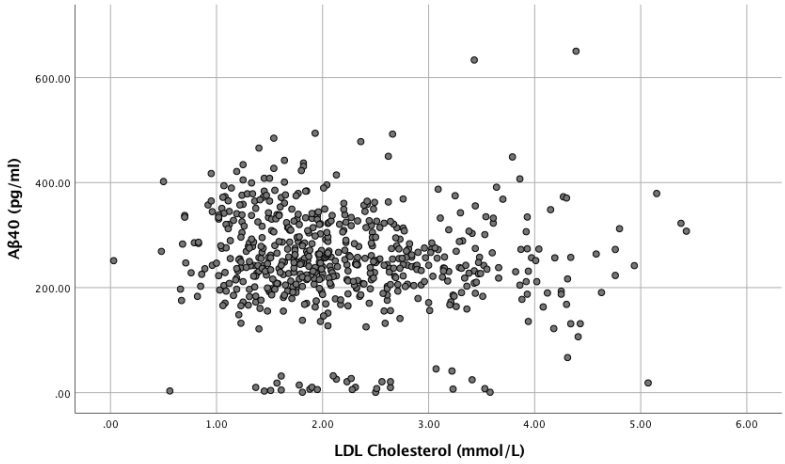

Figure 2-6: Scatter plot of LDL cholesterol and β-amyloid 40 in n=589 subjects, r=0.140, p=0.001.

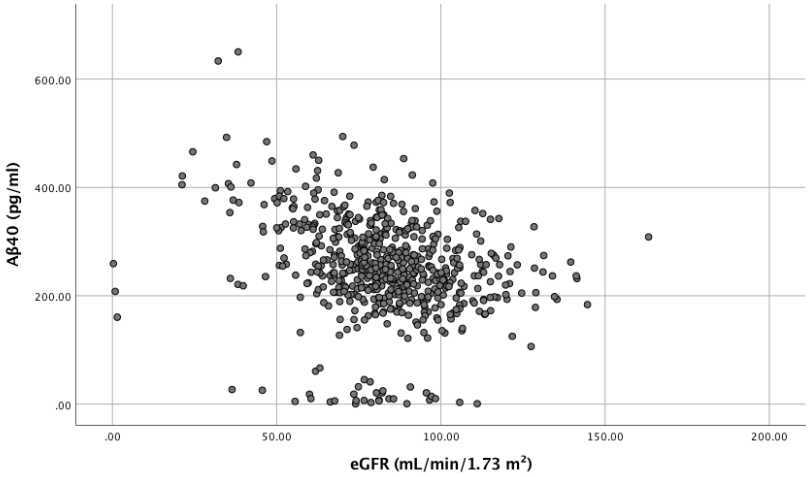

Figure 2-7: Scatter plot of eGFR and β-amyloid 40 in n=483 subjects, r=-0.354, p=1.15E-15.

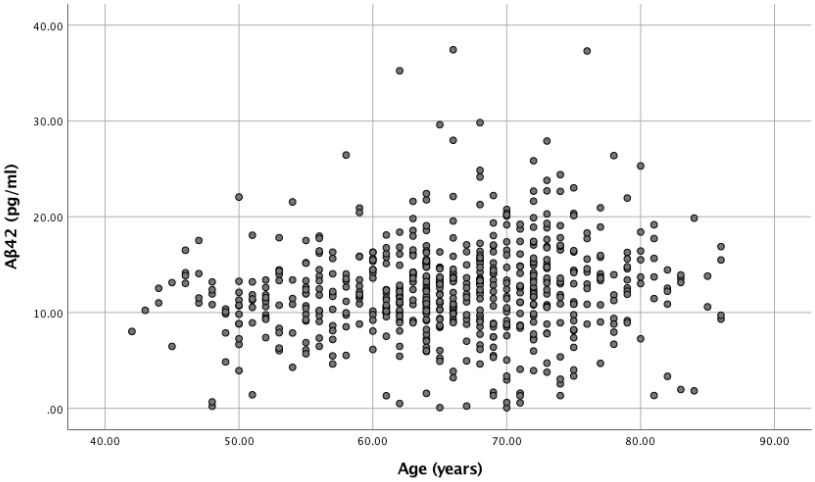

Figure 2-8: Scatter plot of age with β-amyloid 42 in n=628 subjects, r=0.140, p=4.5E-4.

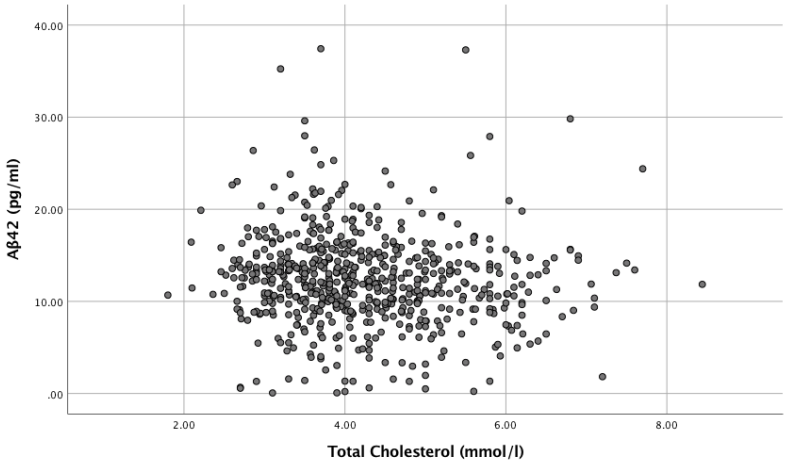

Figure 2-9: Scatterplot of total cholesterol with β-amyloid 42 in n=632 subjects, r=-0.125, p=0.002.

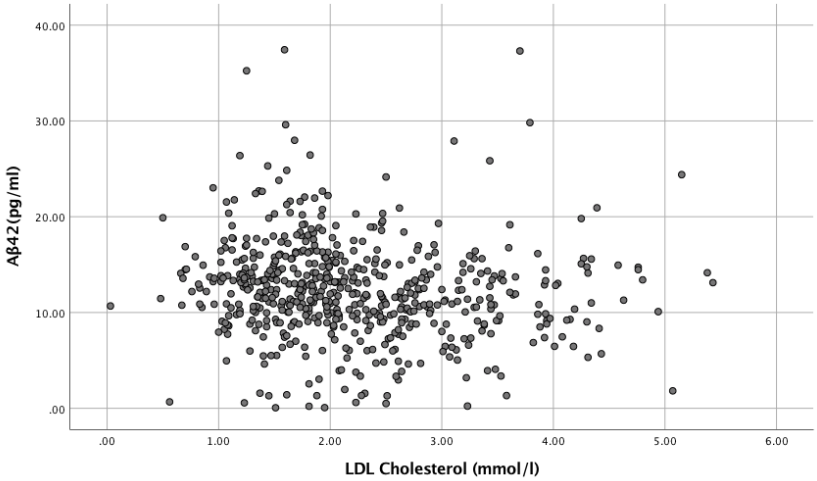

Figure 2-10: Scatter plot of LDL cholesterol with β-amyloid 42 in n=589 subjects, r=-0.179, p=1.2E-5.

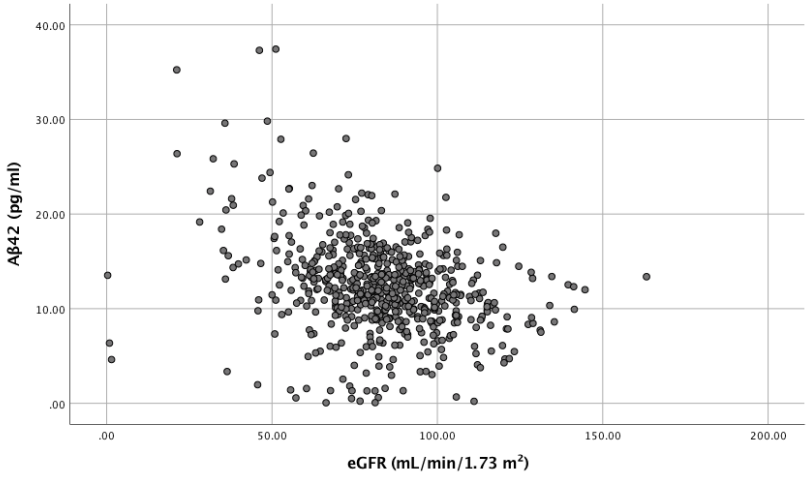

Figure 2-11: Scatter plot of eGFR with β-amyloid 42 in n=482 subjects, r=-0.367, p=8.9E-17.

Based on the above analysis of the baseline SUMMIT cohort, a significant positive correlation exists between β-amyloid 40 and age, while significant negative correlations are present between β-amyloid 40 and height, LDL cholesterol, total cholesterol as well as eGFR. Similarly, for β-amyloid 42, a significant positive correlation was observed with age, and significant negative correlations with LDL cholesterol, total cholesterol and eGFR.

2.5.4 Effect of Binary Determinants on Plasma β-amyloid levels – SUMMIT Cohort

As the first step in exploring the relationship between plasma β-amyloid levels and binary determinants, the distribution of variables was assessed. Due to the distribution of variables, the Mann-Whitney test was use to compare plasma β-amyloid levels. The figures below summarise the results of mean comparisons for the following set of binary determinants: diabetes status, CVD status, gender, ACE inhibitor use, angiotensin II receptor blocker use, B-blocker use, calcium channel blocker use, diuretic use, fibrate use, NSAID use, nitrite use, statin use and steroid use. Results for β-amyloid 40 are presented first followed by β-amyloid 42. With a total of 26 comparisons made, the new value for significance was set at $p<0.002$ at univariate level.

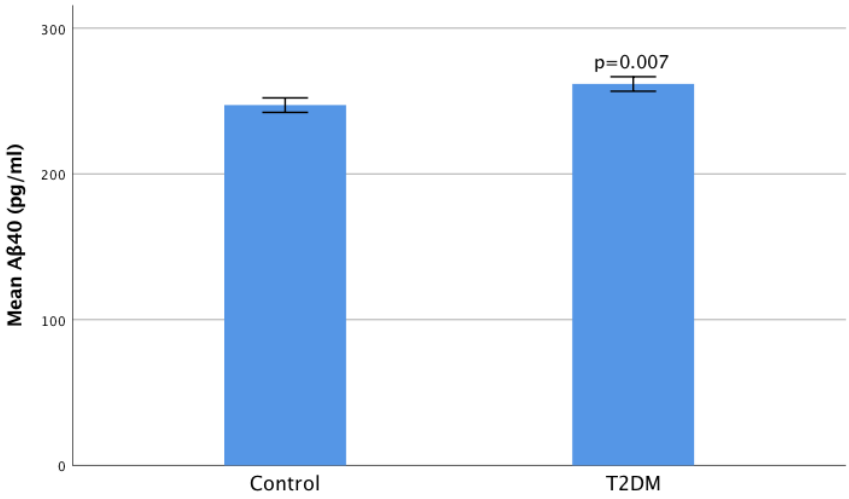

Figure 2-12: Comparison of β-amyloid 40 levels in subjects without and with diabetes, n=244, n=399 respectively.

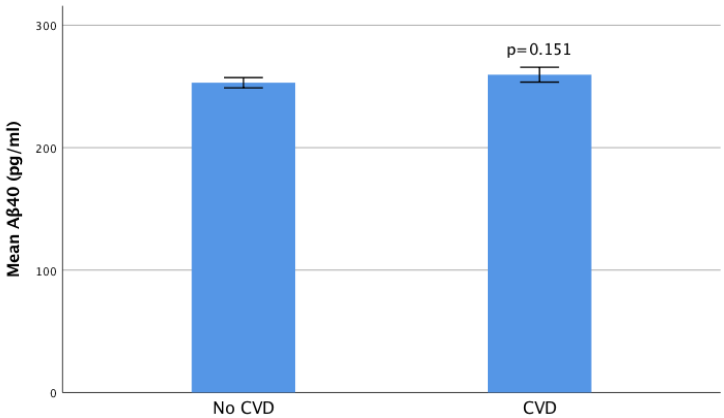

Figure 2-13: Comparison of β-amyloid 40 levels in subjects without and with CVD, n=334, n=309 respectively.

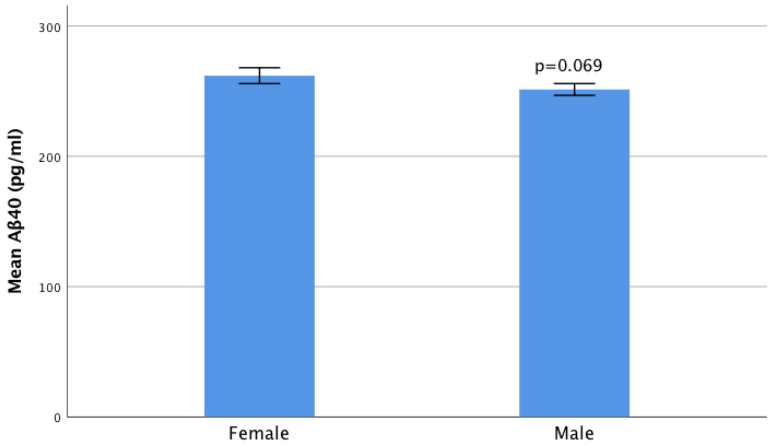

Figure 2-14: Comparison of β -amyloid 40 levels in females and males, n=223, n=405 respectively.

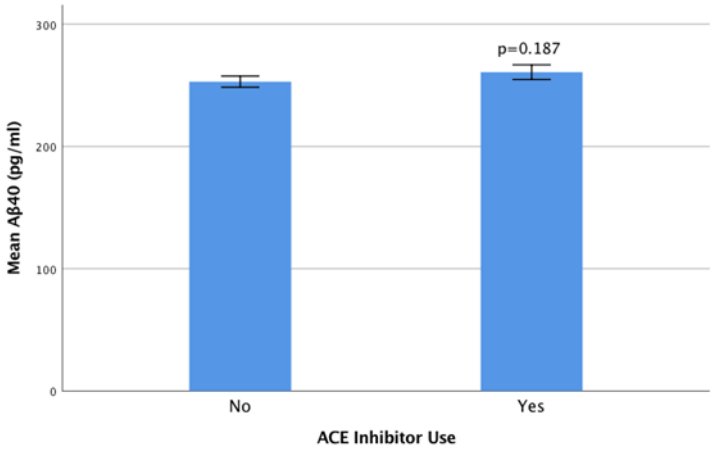

Figure 2-15: Comparison of β-amyloid 40 levels in subjects without and with ACE inhibitor use, n=383, n=256 respectively

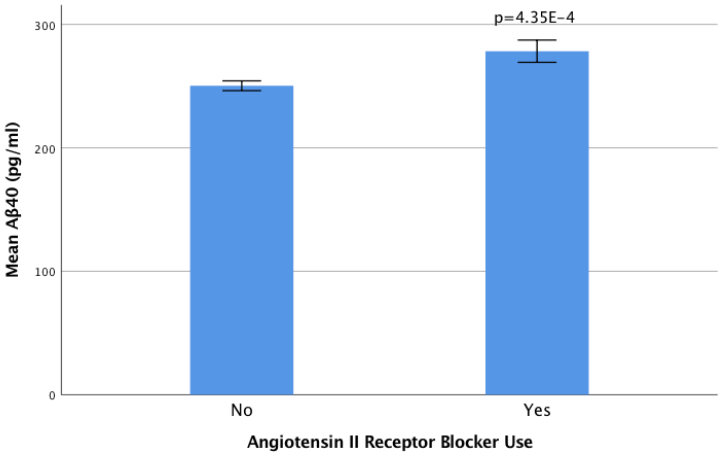

Figure 2-16: Comparison of β-amyloid 40 levels in subjects without and with angiotensin II receptor blocker use, n =515, n=118 respectively.

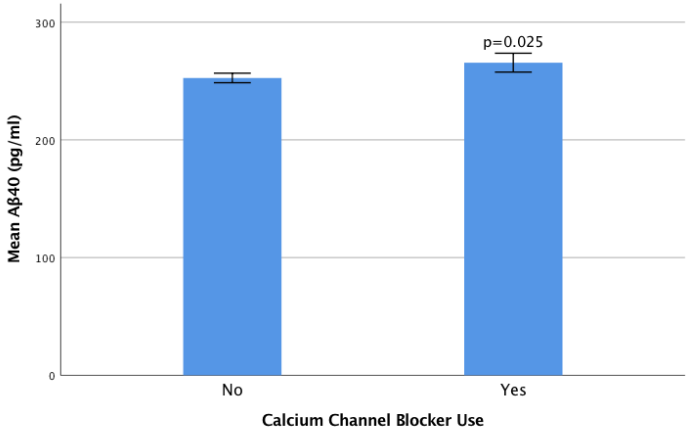

Figure 2-17: Comparison of β-amyloid 40 levels in subjects without and with calcium channel blocker use, n=480, n=190 respectively.

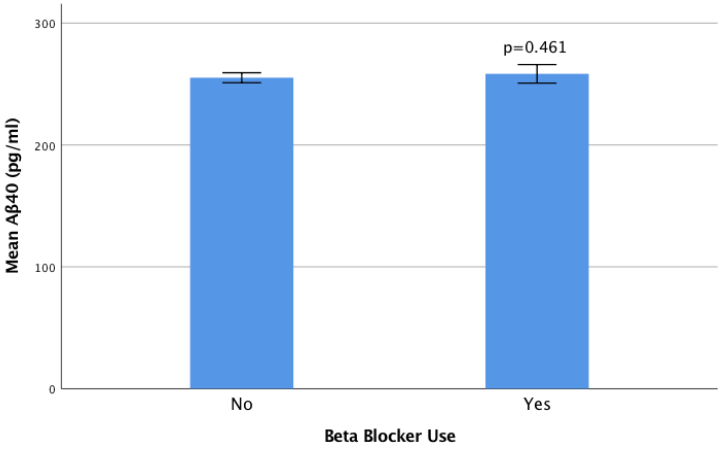

Figure 2-18: Comparison of β-amyloid 40 levels in subjects without and with β-blocker use, n=448, n=190 respectively

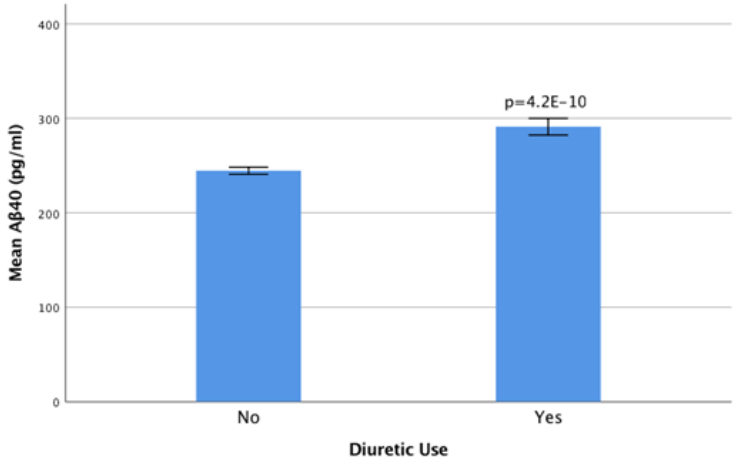

Figure 2-19: Comparison of β-amyloid 40 levels in subjects without and with diuretic use, n=479, n=158 respectively.

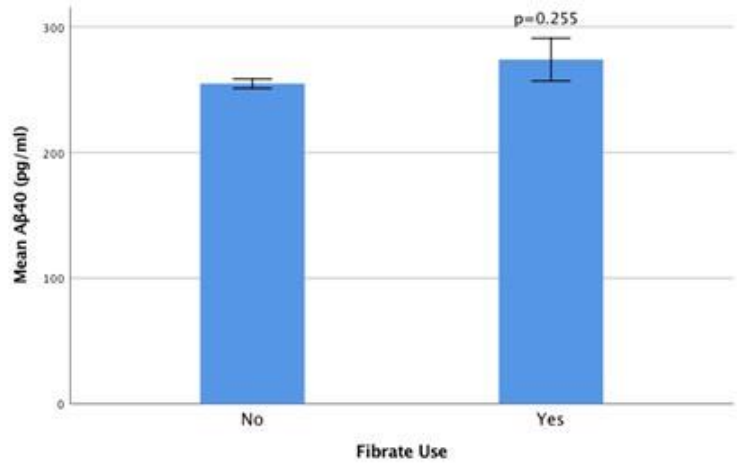

Figure 2-20: Comparison of β-amyloid 40 levels in subjects without and with fibrate use, n =604, n=30 respectively.

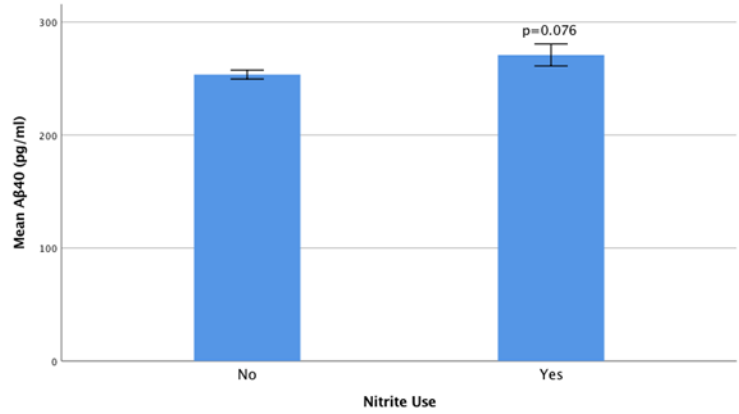

Figure 2-21: Comparison of β-amyloid 40 levels in subjects without and with nitrite use, n=539, n=98 respectively.

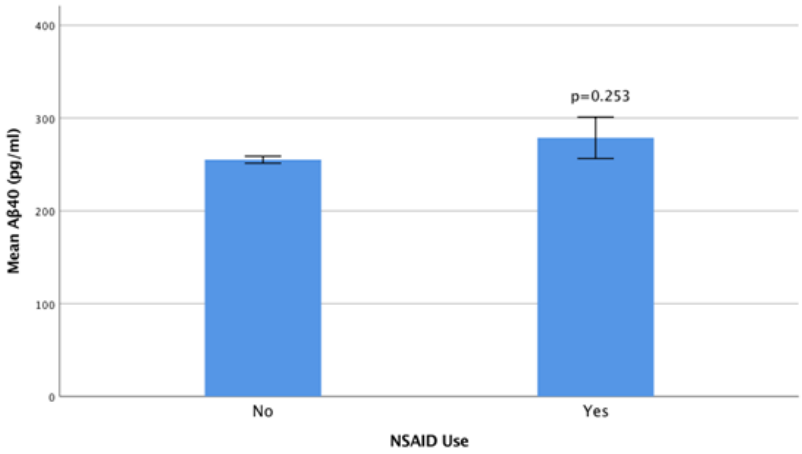

Figure 2-22: Comparison of β-amyloid 40 levels in subjects without and with NSAID use, n=617, n=17 respectively

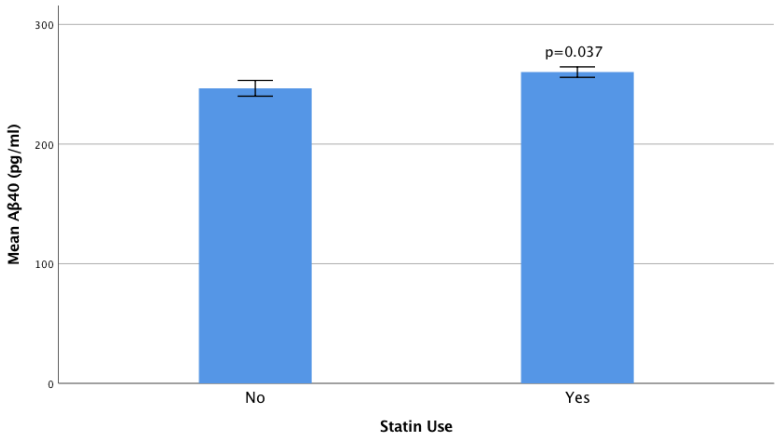

Figure 2-23: Comparison of β-amyloid 40 levels in subjects without and with statin use, n=186, n=452 respectively.

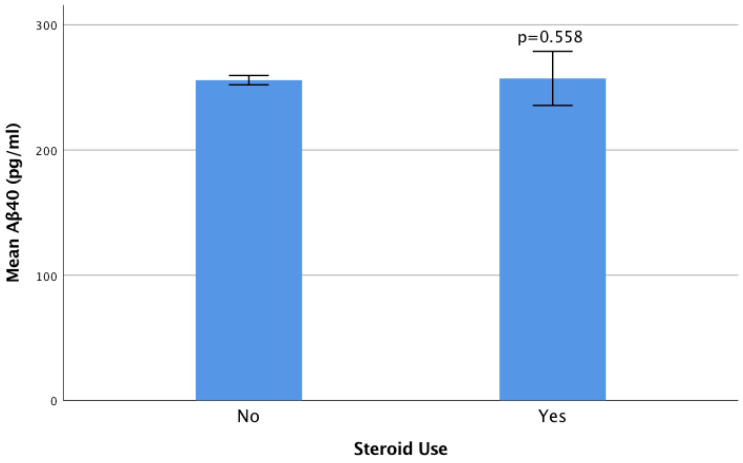

Figure 2-24: Comparison of β-amyloid 40 levels in subjects without and with steroid use, n=611, n=22 respectively.

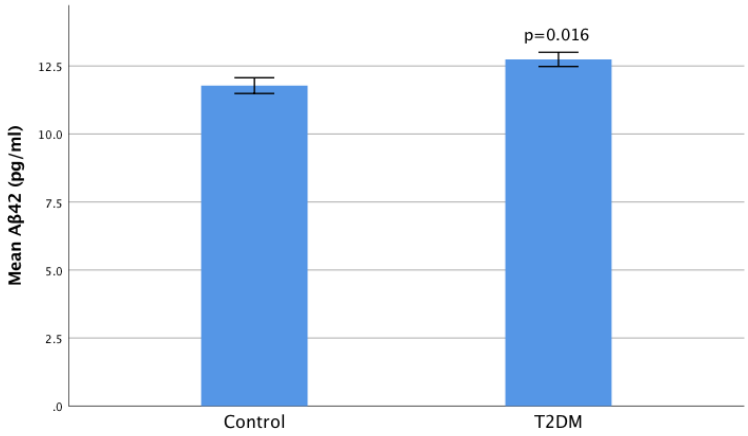

Figure 2-25: comparison of β-amyloid 42 levels in subjects without and with T2DM, n=244, n=399 respectively.

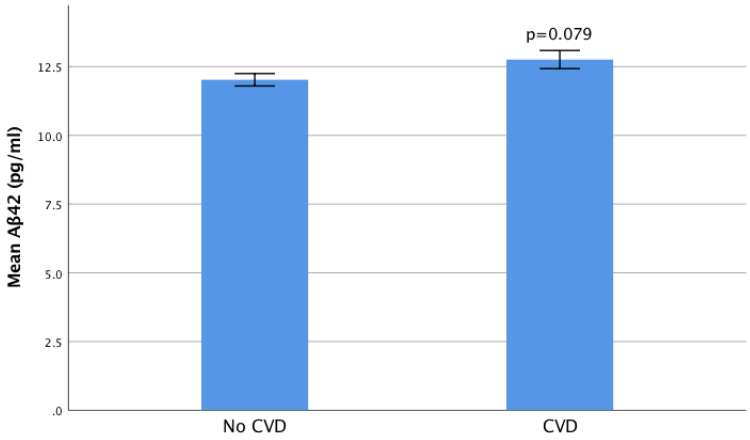

Figure 2-26: Comparison of β-amyloid 42 levels in subjects without and with CVD, n=334, n=309 respectively.

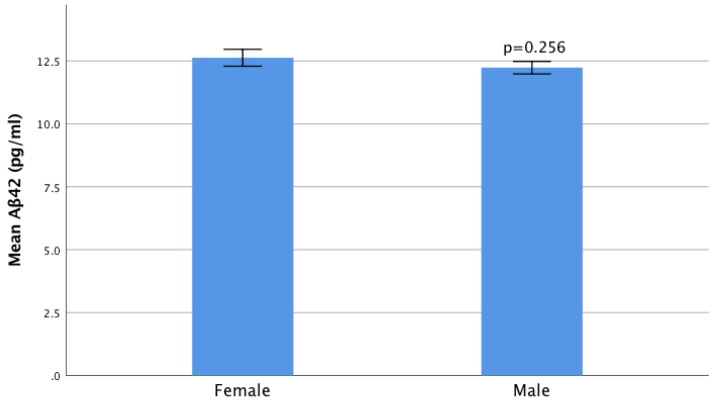

Figure 2-27: Comparison of β-amyloid 42 levels in female and male subjects, n=224, n=404 respectively.

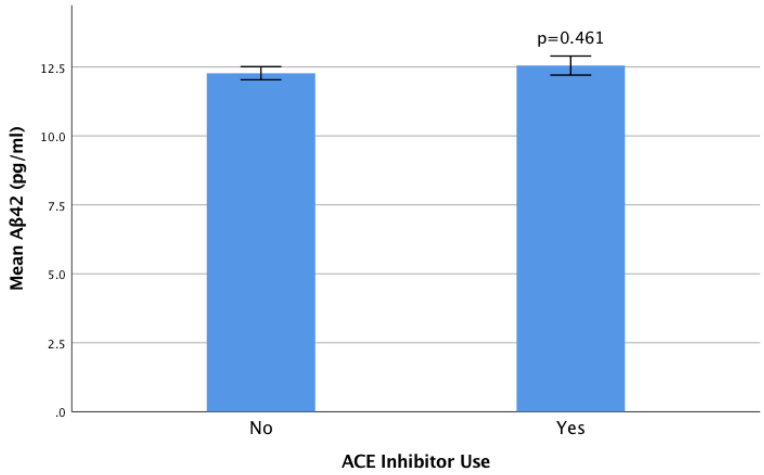

Figure 2-28: Comparison of β-amyloid 42 levels in subjects without and with ACE inhibitor use, n=384, n=255 respectively.

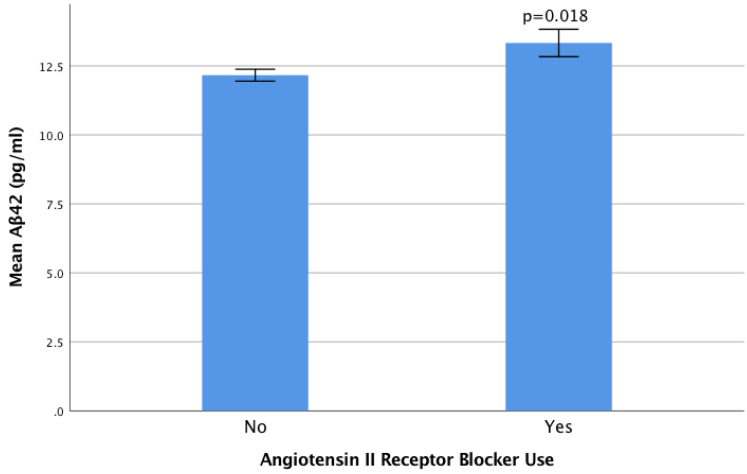

Figure 2-29: Comparison of β-amyloid 42 levels in subjects without and with angiotensin II receptor blocker use, n=516, n=118 respectively.

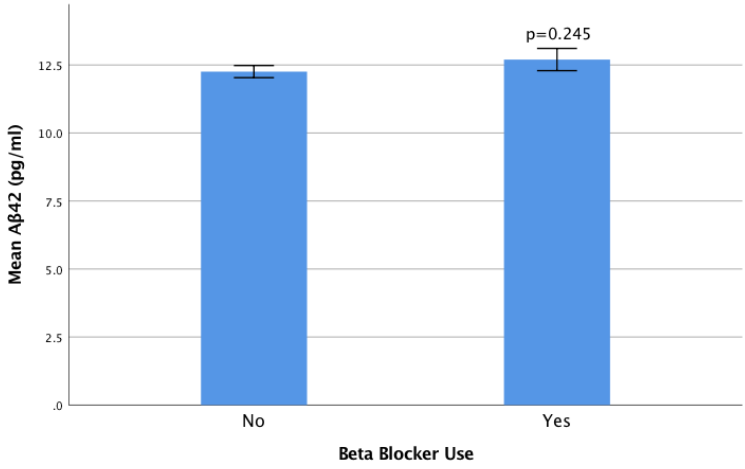

Figure 2-30: Comparison of β-amyloid 42 levels in subjects without and with β-blocker use, n=450, n=188 respectively.

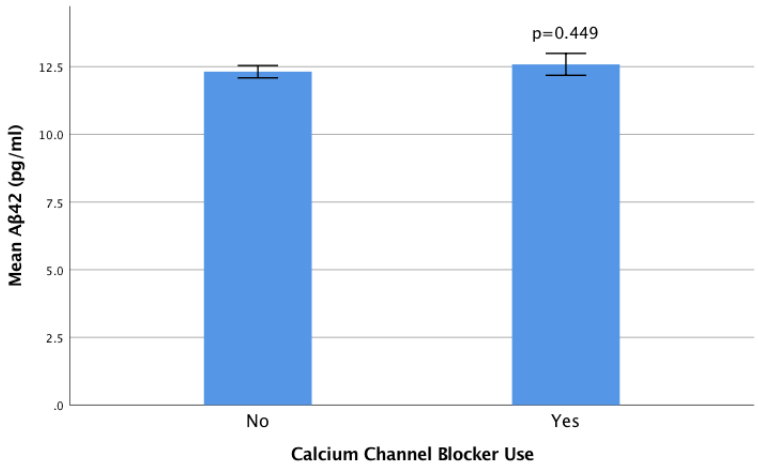

Figure 2-31: Comparison of β-amyloid 42 levels in subjects without and with calcium channel blocker use, n=480, n=158 respectively.

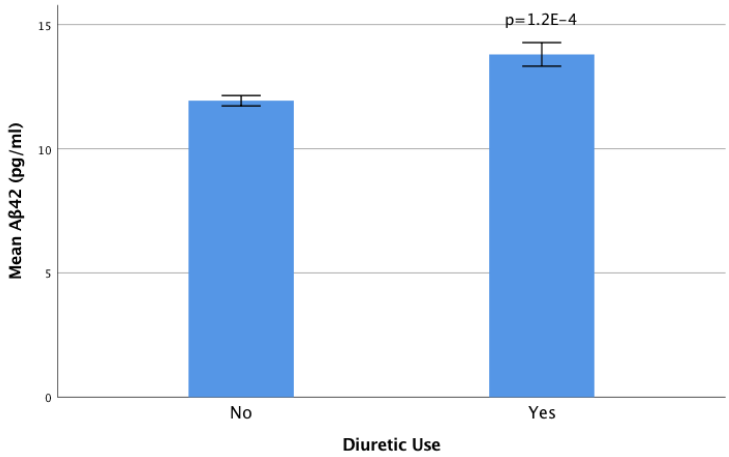

Figure 2-32: Comparison of β-amyloid 42 levels in subjects without and with diuretic use, n=479, n=158 respectively.

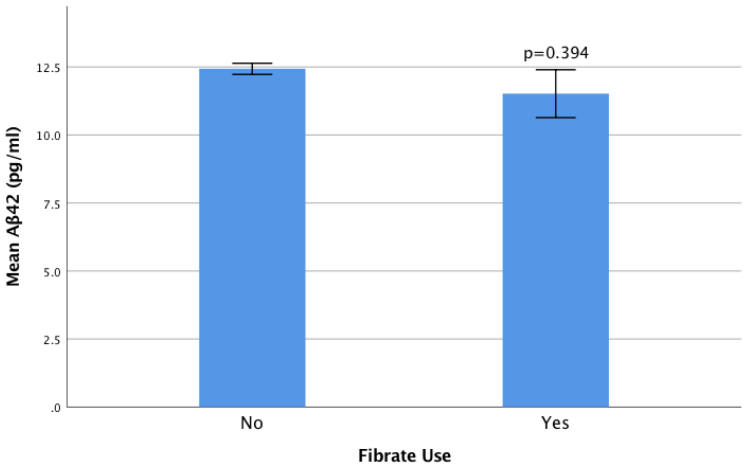

Figure 2-33: Comparison of β-amyloid 42 levels in subjects without and with fibrate use, n=605, n=30 respectively.

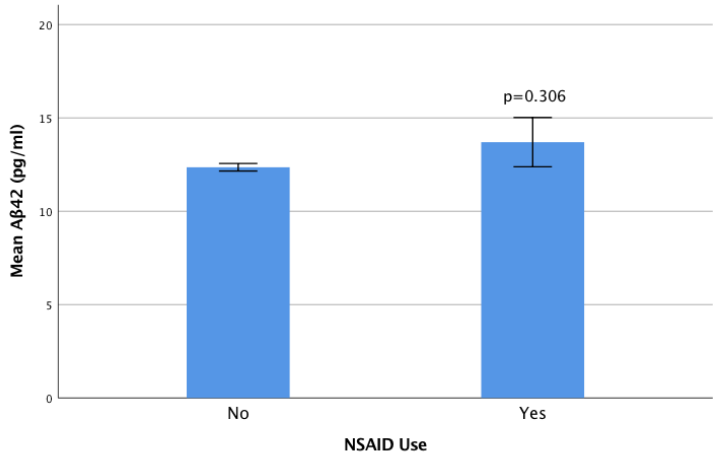

Figure 2-34: Comparison of β-amyloid 42 levels in subjects without and with NSAID use, n=618, n=17 respectively.

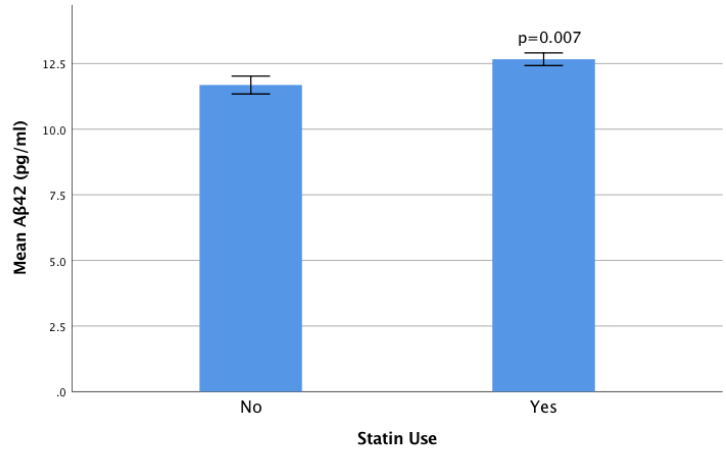

Figure 2-35: Comparison of β-amyloid 42 levels in subjects without and with statin use, n=186, n=452 respectively.

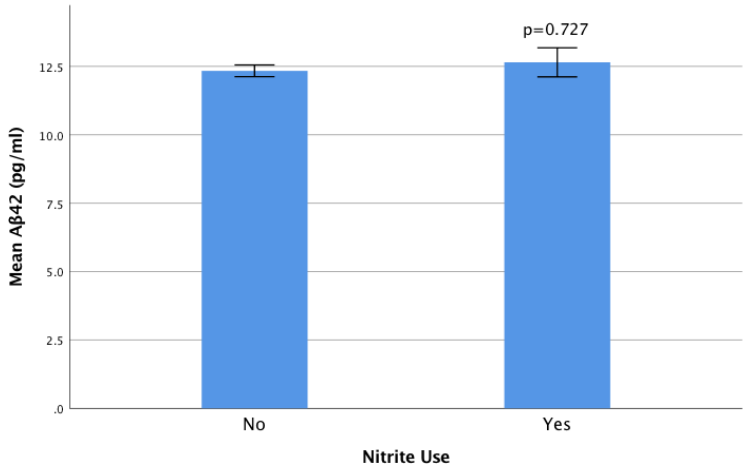

Figure 2-36: Comparison of β-amyloid 42 levels in subjects without and with nitrite use, n=540, n=97 respectively.

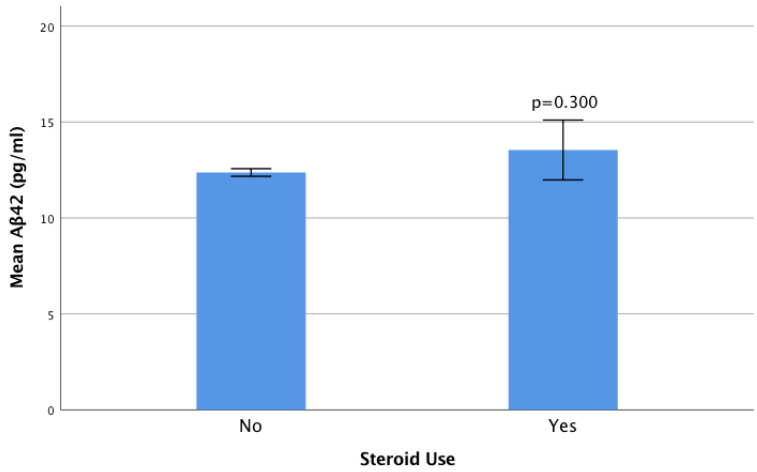

Figure 2-37: Comparison of β-amyloid 42 levels in subjects without and with steroid use, n=622, n=22 respectively.

From the above analysis it can be seen that after adjusting for multiple comparisons, plasma β-amyloid 40 levels are significantly different when stratifying subjects based on angiotensin receptor blocker (ARB) use and diuretic use. β-amyloid 42 levels are significantly different when stratifying the population based on diuretic use. Prior to adjusting for multiple comparisons, other factors that were associated with significantly different levels of β-amyloid 40 at the standard significance level of $p<0.05$ included diabetes status, calcium channel blocker use as well as statin use. At the standard significance value of $p <0.05$, β-amyloid 42 levels were associated with significant differences based on diabetes status, ARB use and statin use.

2.5.5 Regression Analysis of Independent Predictors of β-amyloid - SUMMIT Cohort

In order to determine which factors are independently associated with plasma β-amyloid levels in the SUMMIT baseline cohort, a linear regression model was used. Significant variables identified from the univariate analyses above were entered into the model and selected using forward selection, the p-value cut off for inclusion in the model was set at p=0.05. Forward selection was used to arrive at the final model. B refers to the unstandardized regression coefficient which represents the slope of the model associated with a 1 unit change in the independent variable. Beta-refers to the standardised regression coefficient and allows for direct comparison of the effects of independent variables.

Table 2-3: Regression model, with independent variables age, BMI, diabetes status, height, HbA1c, total cholesterol, LDL cholesterol, HDL cholesterol, eGFR, ARB use, calcium channel blocker use, diuretic use, statin use selected using forward selection.

β-amyloid 40			
Independent Variables	B	Beta	Sig.
eGFR (mL/min/1.73 m²)	-1.007	-0.228	6.26E-08
Diuretic use	34.28	0.157	1.67E-04

Table 2-4: Regression model, with selection independent variables Age, Diabetes status, Height, HbA1c, total cholesterol, LDL cholesterol, eGFR, ARB use, Diuretic use, Statin use selected using forward selection.

β-amyloid 42			
Independent Variables	B	Beta	Sig.
eGFR (mL/min/1.73 m²)	-0.071	-0.289	5.44E-12
LDL Cholesterol (mmol/l)	-0.548	-0.099	0.015
Diuretic Use	1.033	0.085	0.039

As can be seen from these regression models, eGFR and diuretic use were the only significant independent predictors of β-amyloid 40 after adjusting for the factors specified above. For β-amyloid 42, eGFR, LDL cholesterol and diuretic use were the only significant independently associated factors.

2.5.6 Subgroup Analysis: SUMMIT T2DM Cohort

Having looked at factors affecting plasma β-amyloid concentrations in the SUMMIT baseline cohort, the analysis was repeated in diabetes and non-diabetes subgroups. This was done in order to determine whether the same factors are independently associated with plasma β-amyloid in subjects with and without diabetes. Additionally, the effect of different glucose lowering agents on plasma β-amyloid levels could also be investigated. Although diabetes status was not independently associated with plasma β-amyloid levels in the analysis of the SUMMIT baseline cohort, the association between β-amyloid and T2DM processes previously reported in the literature was deemed to be clinically important. Depending on the distribution of variables, either Spearman or Pearson correlation was used in univariate analyses. In order to correct for multiple comparisons, the Bonferroni correction was used, by dividing $p<0.05$ by number of comparisons made. As a result, $p<0.003$ was set as the new threshold for significance. Scatterplots for significant correlations are displayed below.

Table 2-5: Correlation of plasma β-amyloid and baseline continuous characteristics in the SUMMIT T2DM cohort.

Variable	Correlation	Aβ40 (pg/ml)	Aβ42 (pg/ml)
Age (years)	Correlation Coefficient	0.121	0.149
	Sig.	0.017	0.003
	N	385	385
Body Mass Index (kg/m2)	Correlation Coefficient	0.097	0.038
	Sig.	0.056	0.463
	N	385	385
Height (m)	Correlation Coefficient	-0.127	-0.101
	Sig.	0.011	0.045
	N	398	398
Weight (kg)	Correlation Coefficient	0.027	0.006
	Sig.	0.592	0.906
	N	398	398
HbA1c (mmol/mol)	Correlation Coefficient	0.043	0.04
	Sig.	0.394	0.433
	N	391	391
Total Cholesterol (mmol/l)	Correlation Coefficient	-0.129	-0.095
	Sig.	0.01	0.059
	N	393	393

LDL Cholesterol (mmol/l)	Correlation Coefficient	-0.17	-0.166
	Sig.	**0.001**	**0.002**
	N	364	364
HDL Cholesterol (mmol/l)	Correlation Coefficient	-0.099	-0.036
	Sig.	0.05	0.477
	N	390	390
Triglycerides (mmol/l)	Correlation Coefficient	0.088	0.075
	Sig.	0.085	0.141
	N	386	386
eGFR (mL/min/1.73 m²)	Correlation Coefficient	-0.301	-0.304
	Sig.	**2.67E-09**	**1.88E-09**
	N	375	375

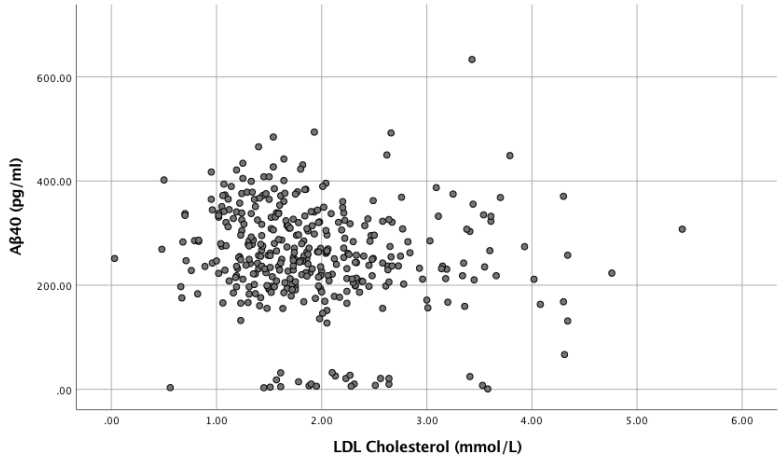

Figure 2-38: Scatter plot of LDL cholesterol and plasma β-amyloid 40 in the SUMMIT T2DM cohort. n=364, r=-0.17, p=0.001.

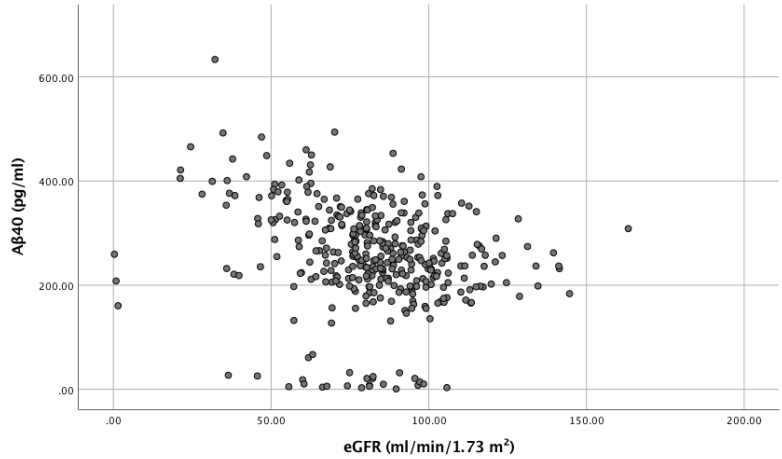

Figure 2-39: Scatter plot of eGFR and β-amyloid 40 – SUMMIT T2DM cohort. n= 375, r=-0.301, p=2.67E-9.

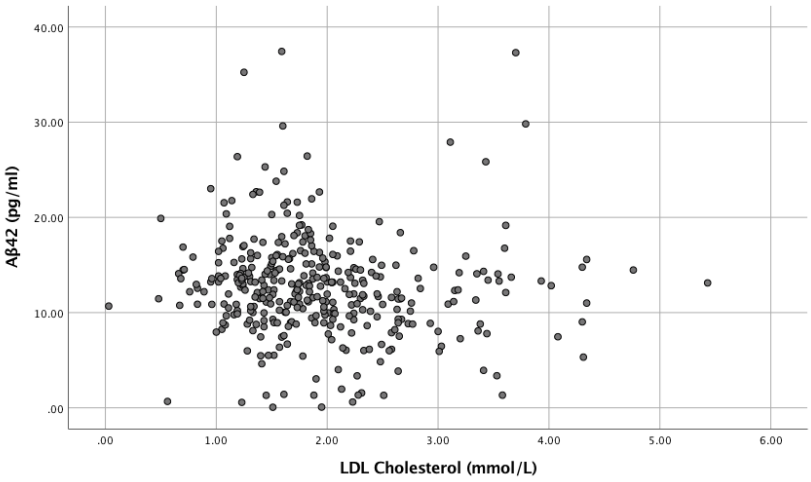

Figure 2-40: Scatter plot of LDL cholesterol and plasma β-amyloid 42 – SUMMIT T2DM cohort. n=364, r=-0.166, p=0.002.

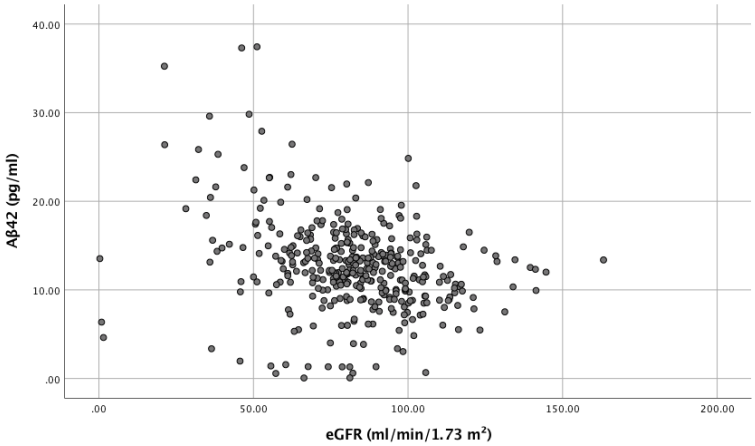

Figure 2-41: Scatter plot of eGFR and β-amyloid 42 – SUMMIT T2DM cohort. n=375, r=-0.304, p=1.88E-9.

The above analysis of continuous determinants of plasma β-amyloid revealed that in the T2DM cohort, a significant negative correlation is exists between both β-amyloid 40 and 42 with LDL cholesterol as well as eGFR.

2.5.7 Effect of Binary Determinants on Plasma β-amyloid Levels – SUMMIT T2DM cohort

To analyse the association of different pharmacological agents with plasma β-amyloid in the diabetes cohort, new plasma β-amyloid quartiles were calculated using only the values of diabetes subjects. In addition to medications analysed in the baseline cohort, glucose lowering therapies were also analysed. These included biguanides, dipeptidyl peptidase 4 inhibitors, glitazones, incretins, insulin and sulphonylureas. For the comparisons below, a p value of <0.002 was set as the new threshold for statistical significance in order to adjust for multiple comparisons.

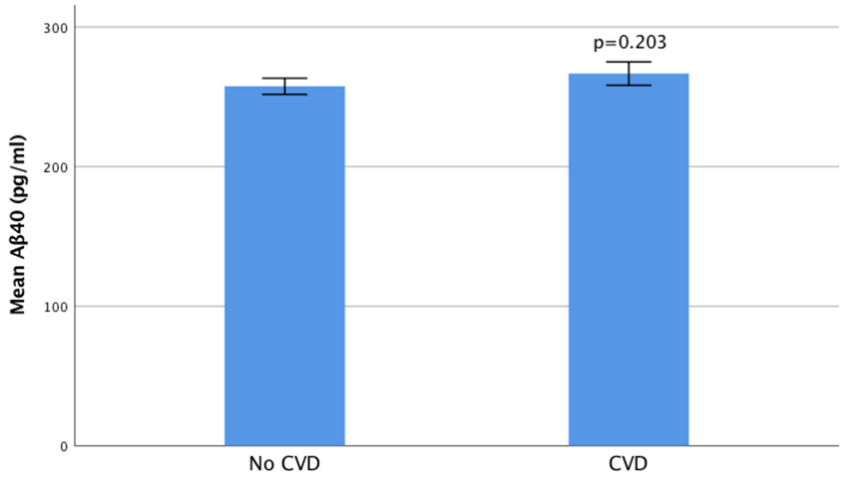

Figure 2-42: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with CVD, n=214, n=185 respectively.

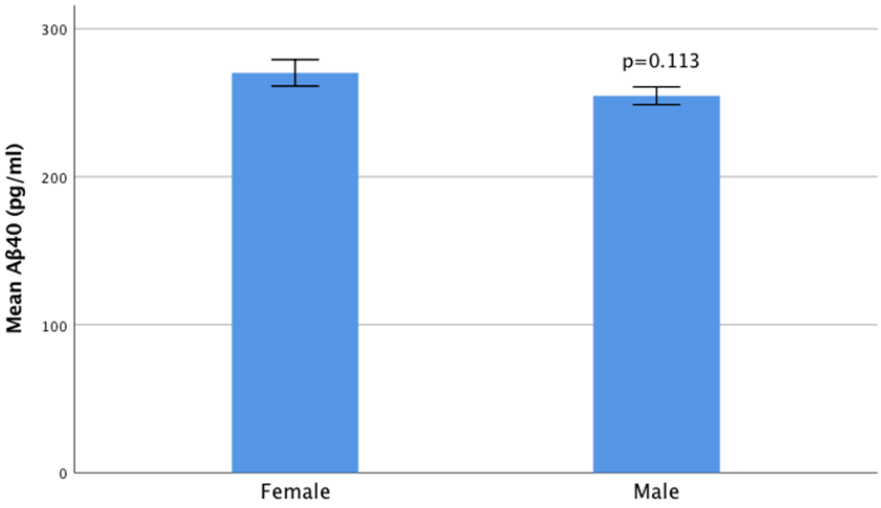

Figure 2-43: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in females and males, n=127, n=258 respectively.

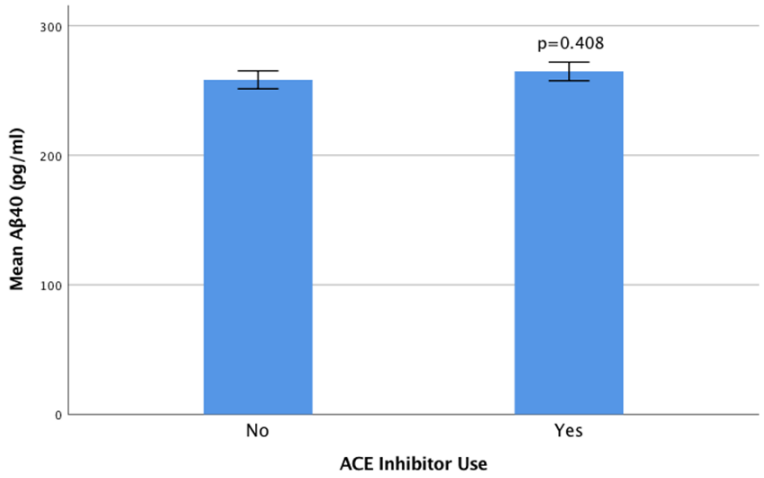

Figure 2-44: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with ACE inhibitor use, n=203, n=194 respectively.

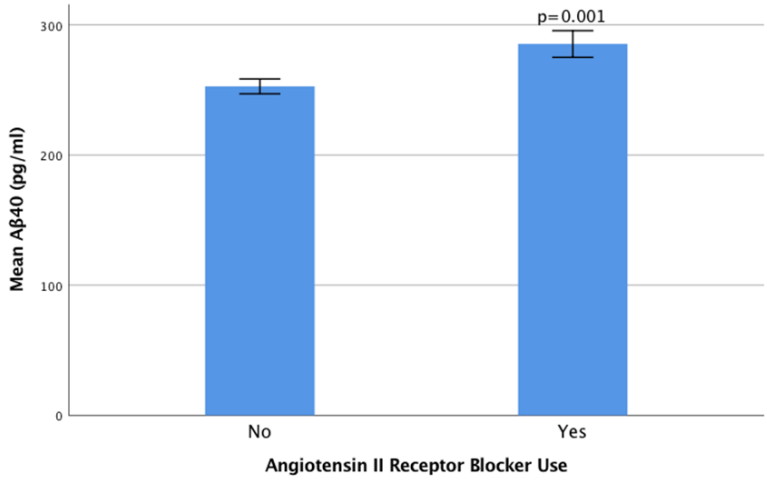

Figure 2-45: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with angiotensin II receptor blocker use, n=297, n=94 respectively.

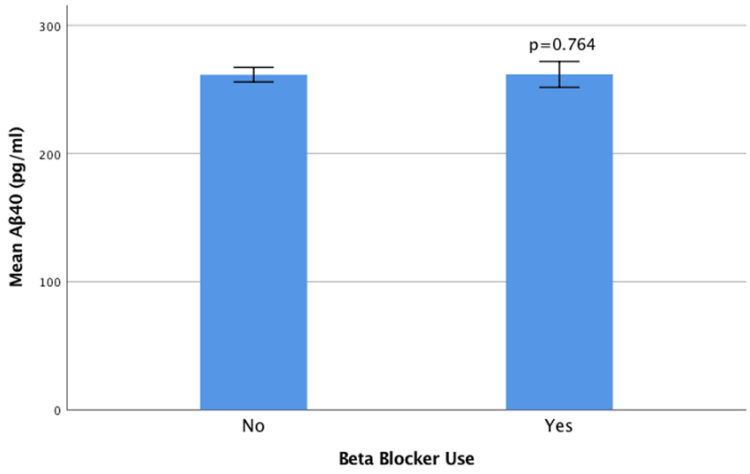

Figure 2-46: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with β-blocker use, n=270, n=125 respectively.

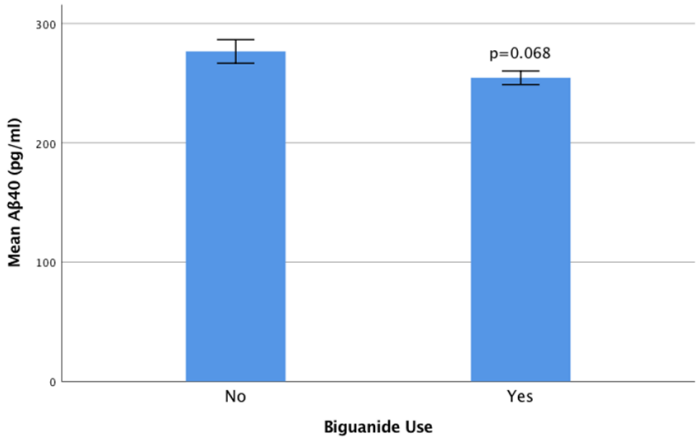

Figure 2-47:SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with biguanide use, n=116, n=281 respectively.

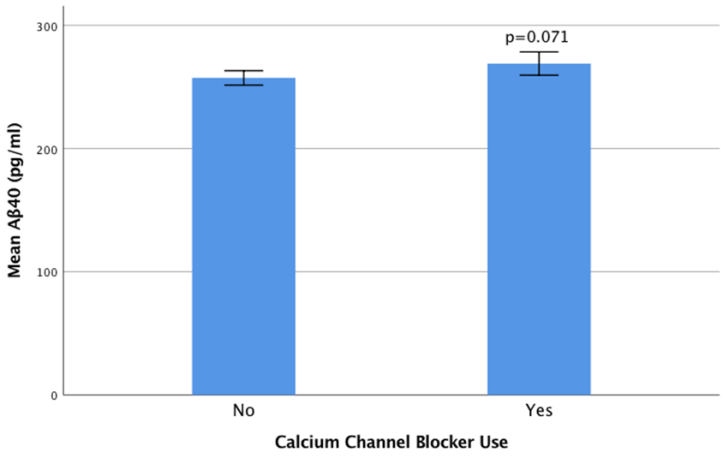

Figure 2-48: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with calcium channel blocker use, n=281, n=114 respectively.

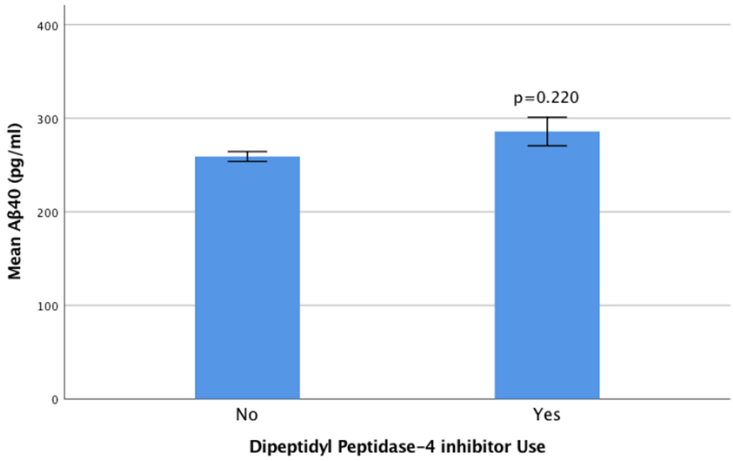

Figure 2-49: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with DPP-4 inhibitor use, n=367, n=26 respectively.

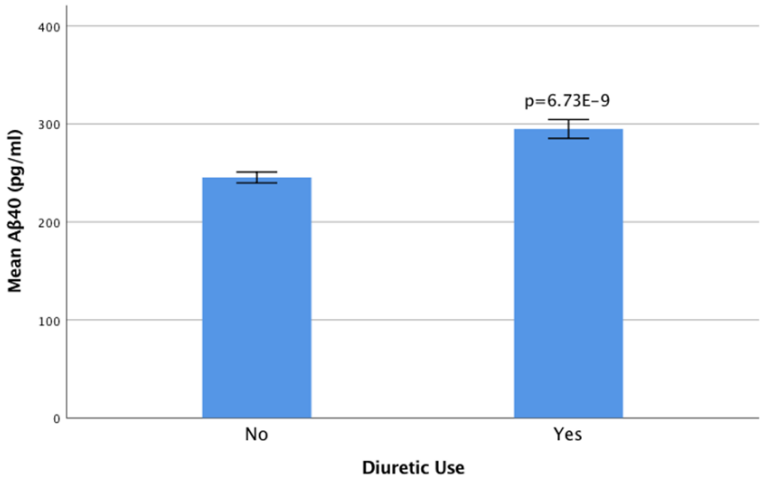

Figure 2-50: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with diuretic use, n=266, n=129 respectively.

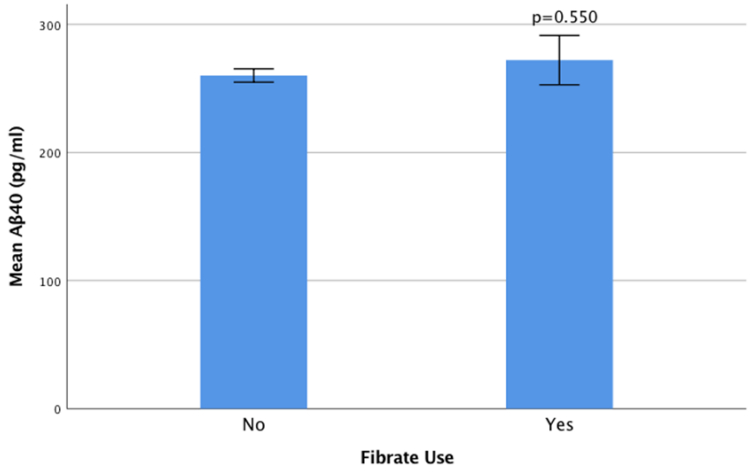

Figure 2-51: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with fibrate use, n=367, n=25 respectively.

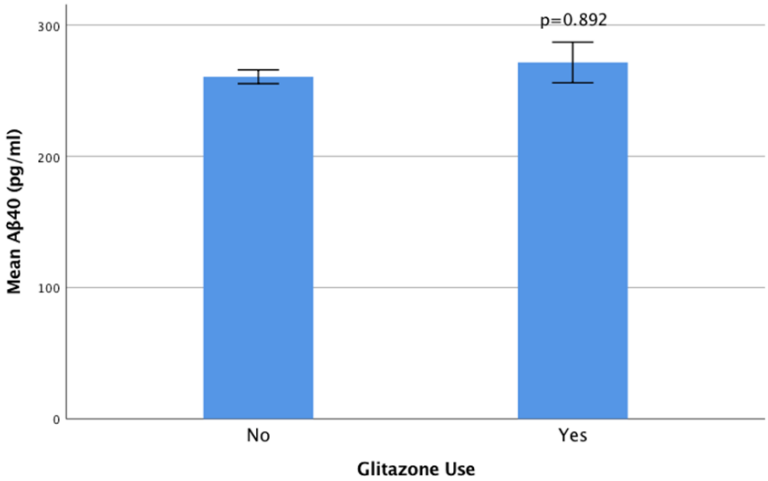

Figure 2-52: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with glitazone use, n=368, n=25 respectively.

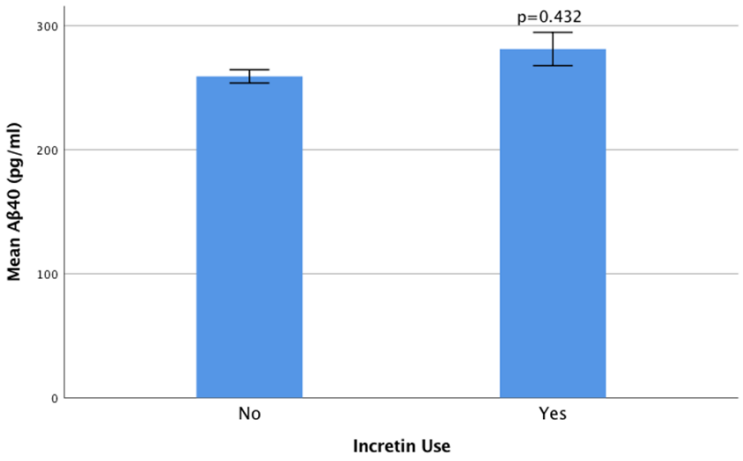

Figure 2-53: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with incretin use, n=359, n=36 respectively.

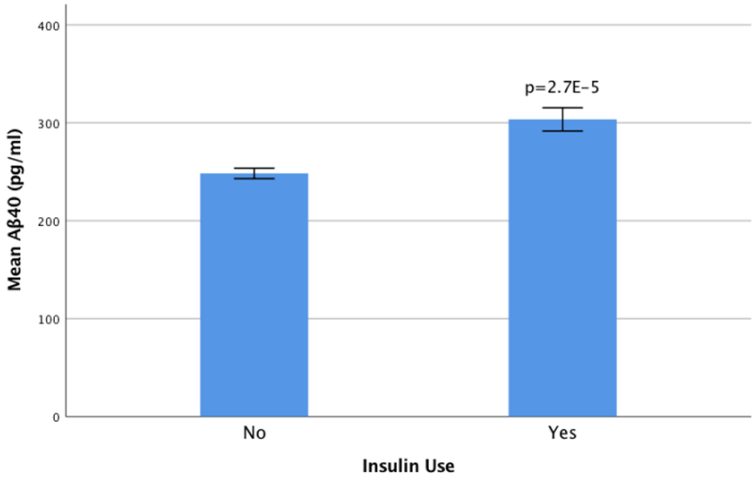

Figure 2-54: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with insulin use, n=302, n=92 respectively.

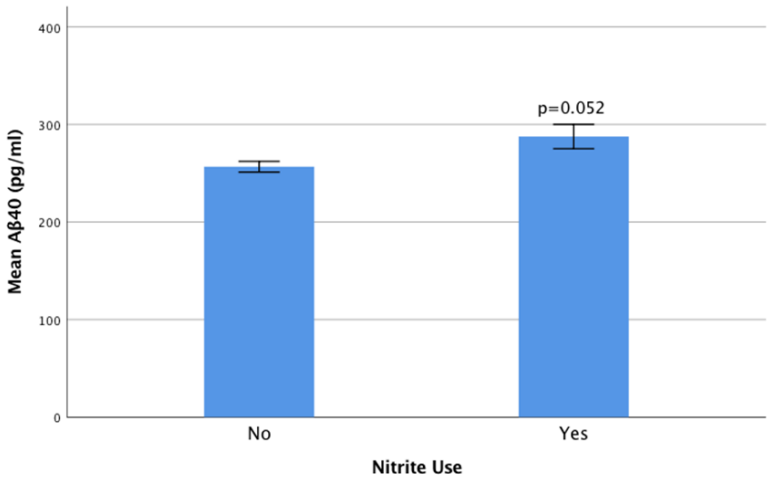

Figure 2-55: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with nitrite use, n=331, n=63 respectively.

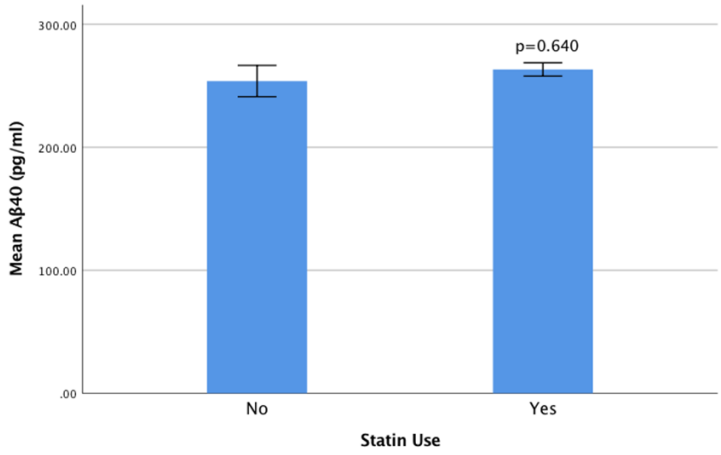

Figure 2-56: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with statin use, n=73, n=324 respectively.

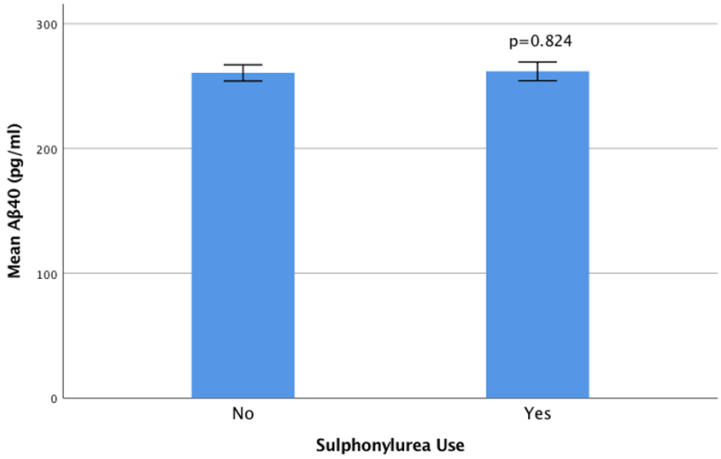

Figure 2-57: SUMMIT T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with sulphonylurea use, n=266, n=131 respectively.

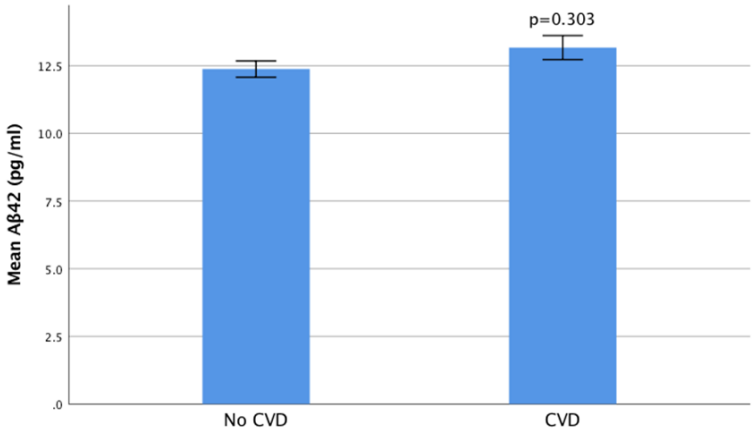

Figure 2-58: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with CVD, n=214, n=185 respectively.

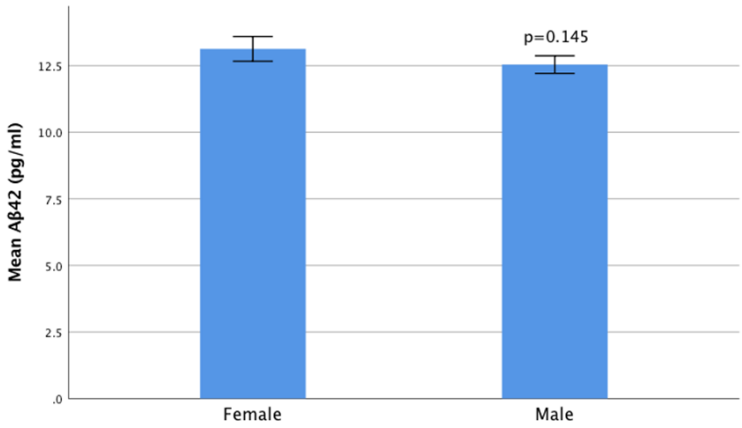

Figure 2-59: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in female and male subjects, n=127, n=258 respectively.

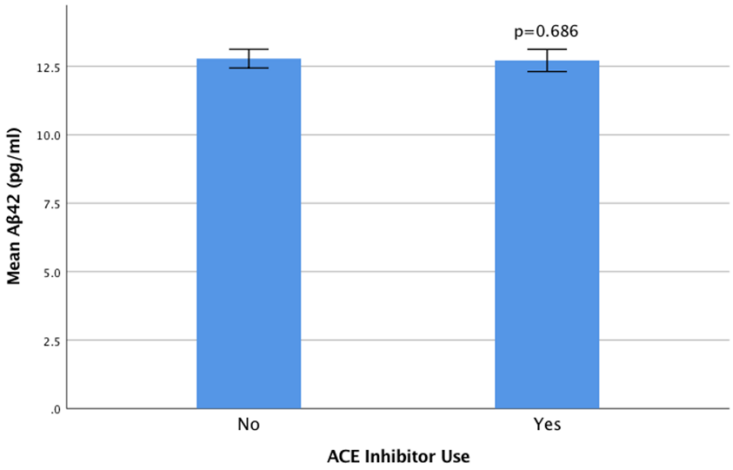

Figure 2-60: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with ACE inhibitor use, n=204, n=193.

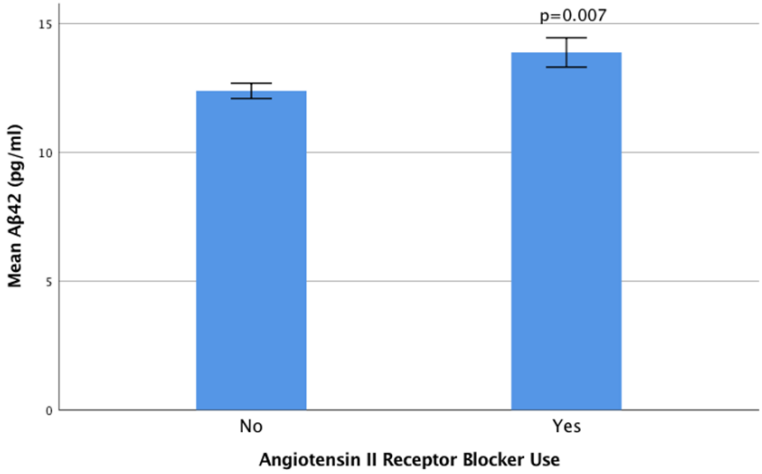

Figure 2-61: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with angiotensin II receptor blocker use , n=297, n=95 respectively.

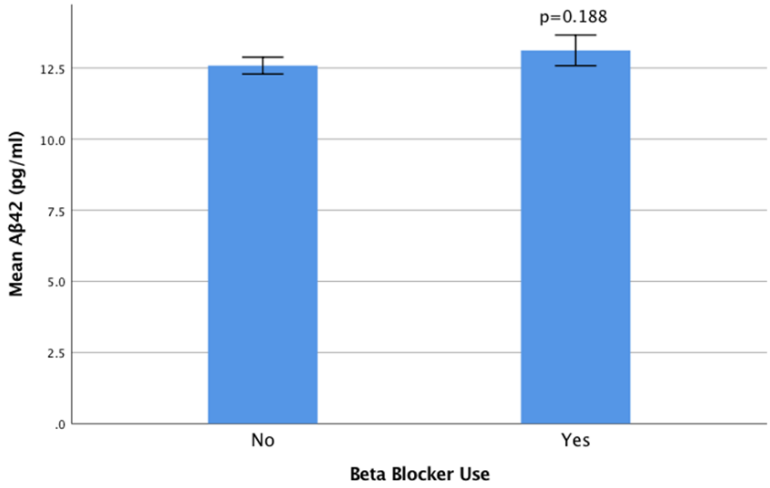

Figure 2-62: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with β-blocker use, n=271, n=124 respectively.

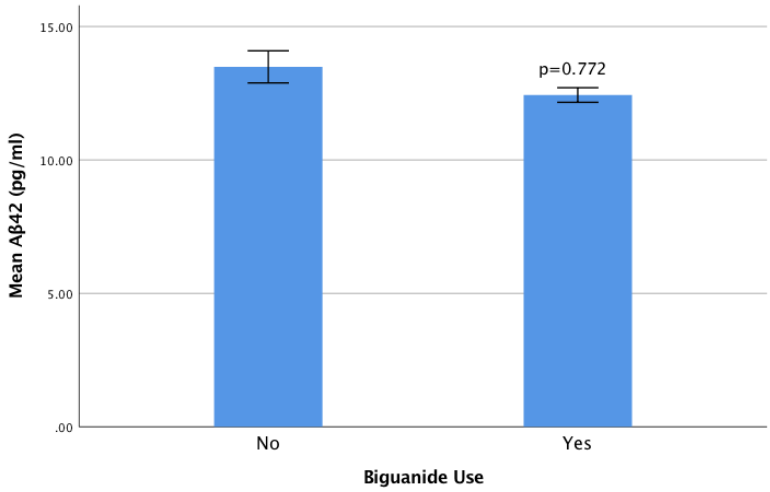

Figure 2-63: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with biguanide use, n=117, n=281 respectively.

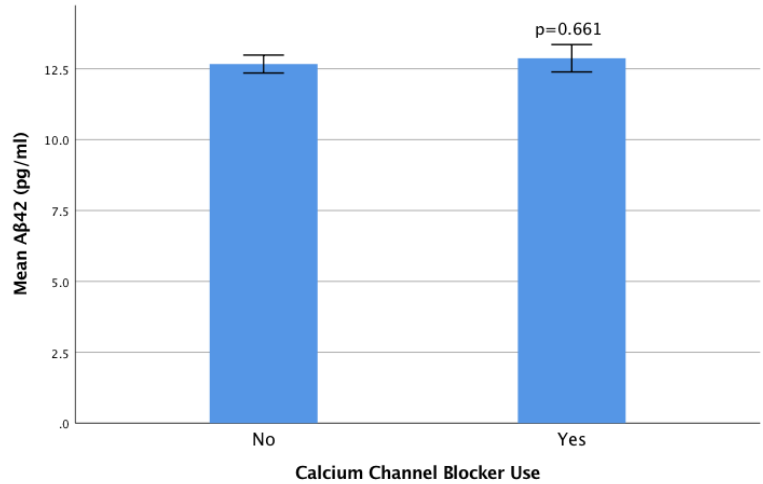

Figure 2-64: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with calcium channel blocker use, n=281, n=115 respectively.

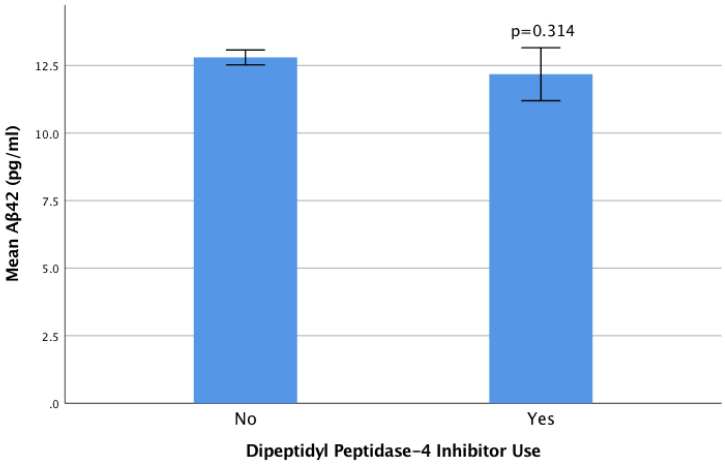

Figure 2-65: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with DPP-4 Inhibitor use, n=368, n=26 respectively.

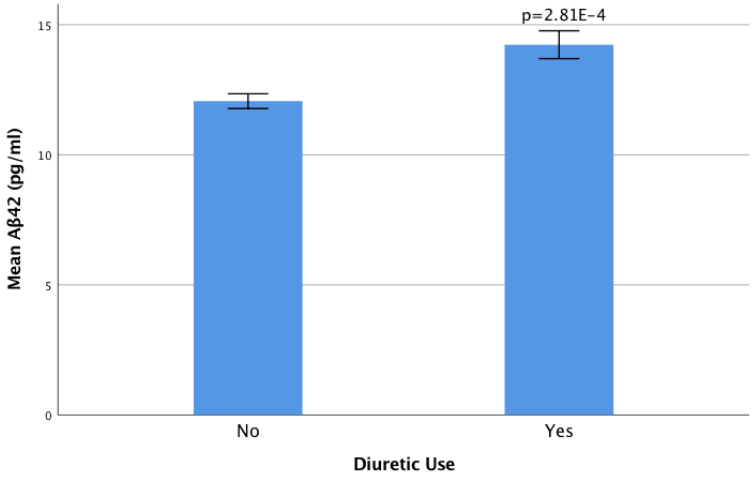

Figure 2-66: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with diuretic use, n=266, n=129 respectively.

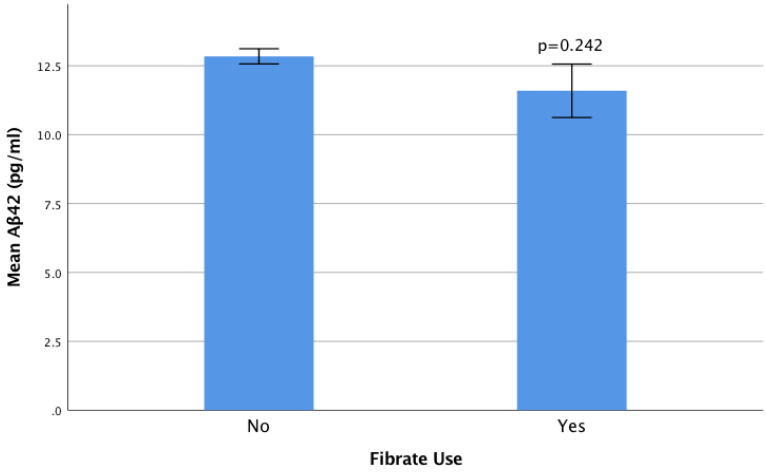

Figure 2-67: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with fibrate use, n=368, n=25 respectively.

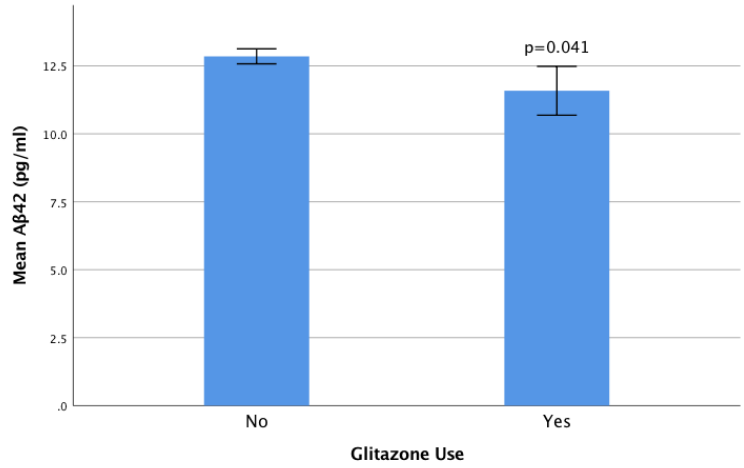

Figure 2-68: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with glitazone use, n=369, n=25 respectively.

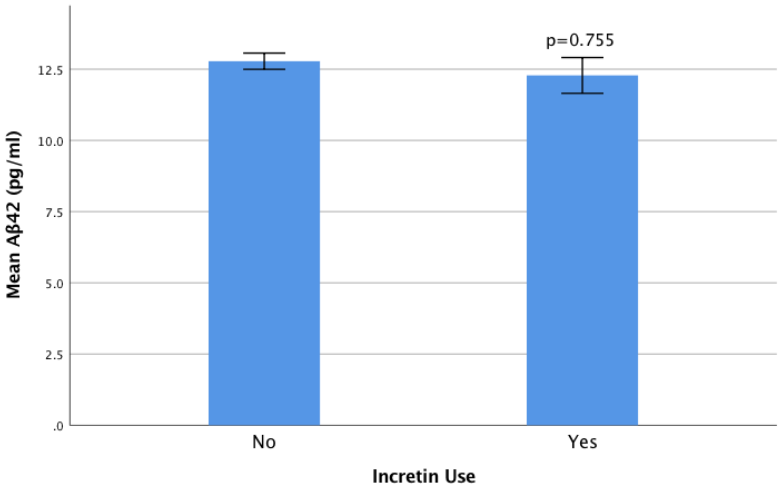

Figure 2-69: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with incretin use, n=360, n=36 respectively.

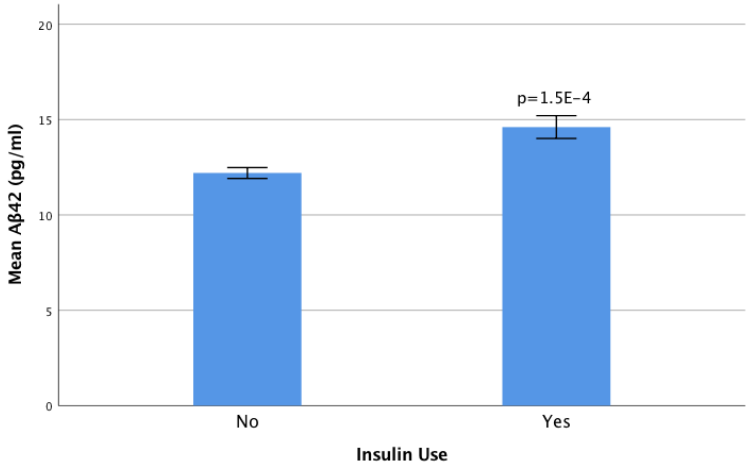

Figure 2-70: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with insulin use, n=303, n=91 respectively.

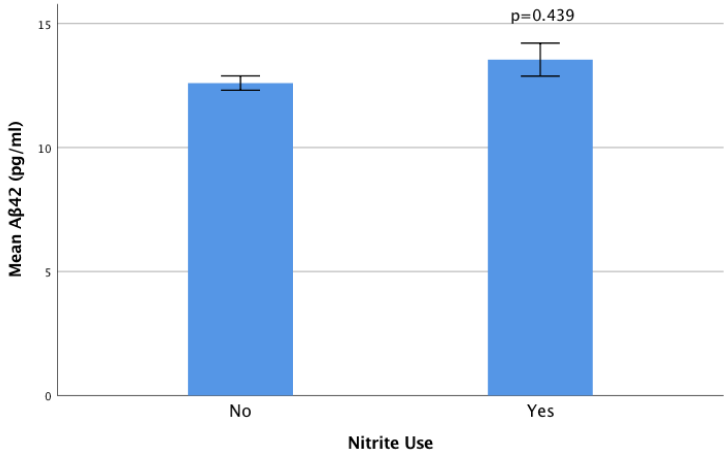

Figure 2-71: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with nitrite use, n=331, n=63 respectively.

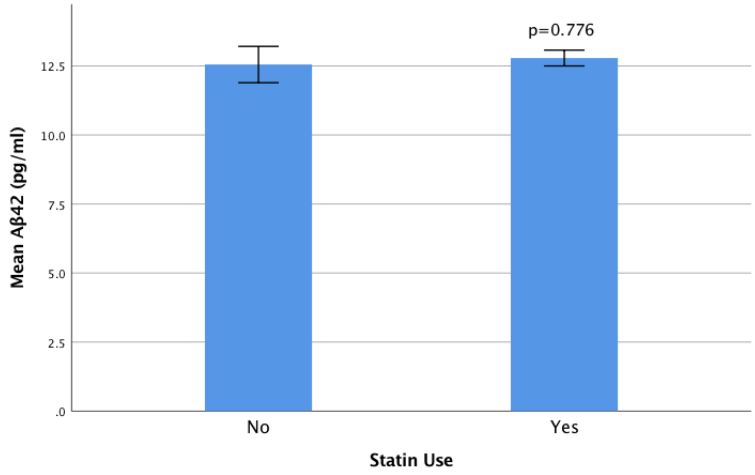

Figure 2-72: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with statin use, n=73, n=324 respectively.

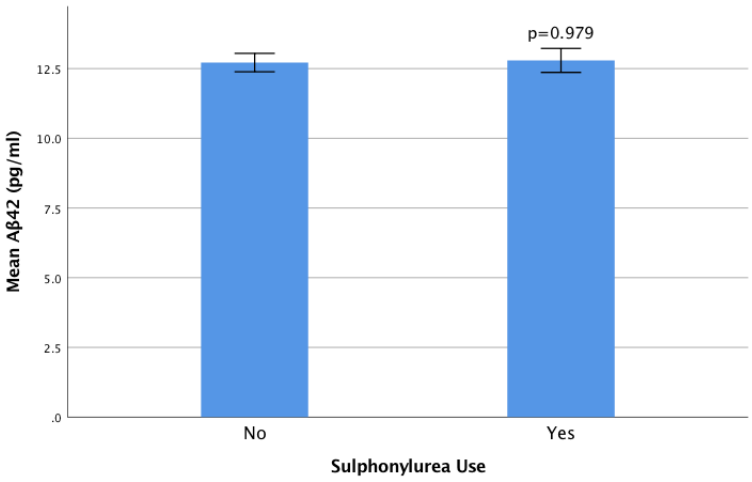

Figure 2-73: SUMMIT T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with sulphonylurea use, n=267, n=131 respectively.

The above analysis looked at binary determinants of β-amyloid 40 and 42, including the use glucose lowering agents. Significant differences in plasma β-amyloid 40 levels were found when dividing subjects based ARB use, diuretic use and insulin use. When looking at β-amyloid 42, significant differences were found when dividing subjects based on diuretic use and insulin use.

2.5.8 Regression Analysis of Independent Determinants of β-amyloid – SUMMIT T2DM Cohort

In order to determine which factors are independently associated with plasma β-amyloid levels in diabetic subjects, a linear regression model was used. The standard threshold for significance ($p<0.05$) was used to select factors to be included in the model, as the linear regression method adjusts for multiple comparisons Significant variables identified from the analyses above were entered into the model and selected using forward selection.. B refers to the unstandardized regression coefficient which represents the slope of the model associated with a 1 unit change in the independent variable. Beta refers to the standardised regression coefficient and allows for direct comparison of the effects of independent variables.

Table 2-6: Linear regression model with and independent variables Age, Height, Total cholesterol, LDL Cholesterol, eGFR, Insulin use, Diuretic use, ARB use selected using forward selection – SUMMIT T2DM cohort.

β-amyloid 40			
Independent Variables	B	Beta	Sig.
Insulin Use	47.089	0.195	3.35E-4
eGFR (mL/min/1.73 m²)	-0.780	0.180	0.001
Diuretic Use	28.906	0.135	0.013

Table 2-7: Linear regression model with and independent variables Age, Height, LDL cholesterol, Insulin use, Glitazone use, Diuretic use, ARB use selected using forward selection – SUMMIT T2DM cohort.

β-amyloid 42			
Independent Variables	B	Beta	Sig.
eGFR (mL/min/1.73 m²)	-0.064	-0.266	5.99E-7
Insulin Use	2.303	0.173	0.001

Based on this analysis, it would appear that insulin use, eGFR and diuretic use are the only significant factors independently associated with plasma β-amyloid 40 in the T2DM cohort. For beta amyloid 42, eGFR and insulin use were found to be independent significant determinants.

2.5.9 Subgroup Analysis: SUMMIT No T2DM cohort

The same analysis was then carried out in subjects without T2DM. Pearson or Spearman correlation was used depending on variable distribution. As before, the Bonferroni correction was used to take into account multiple comparisons, with $p<0.003$ as the new accepted threshold for statistical significance. Scatter plots of significant correlations are displayed below.

Table 2-8: Correlations of plasma β-amyloid with continuous baseline characteristics in the SUMMIT non-T2DM cohort.

	Spearman's Rho	Aβ40	Aβ42
Age (years)	Correlation Coefficient	0.281	0.115
	Sig.	**8.0E-06**	0.073
	N	243	243
Body Mass Index (kg/m2)	Correlation Coefficient	0.046	-0.048
	Sig.	0.48	0.454
	N	243	243
Height (m)	Correlation Coefficient	-0.222	-0.122
	Sig.	**4.8E-04**	0.058
	N	244	244
Weight (kg)	Correlation Coefficient	-0.109	-0.127
	Sig.	0.09	0.048
	N	244	244
HbA1c (mmol/mol)	Correlation Coefficient	0.093	0.072
	Sig.	0.149	0.269
	N	241	241
Total Cholesterol (mmol/l)	Correlation Coefficient	-0.049	-0.111
	Sig.	0.449	0.088
	N	239	239
LDL Cholesterol (mmol/l)	Correlation Coefficient	-0.049	-0.151
	Sig.	0.463	0.024
	N	225	225
HDL Cholesterol (mmol/l)	Correlation Coefficient	-0.016	0.07
	Sig.	0.809	0.286
	N	236	236

Triglycerides (mmol/l)	Correlation Coefficient	0.007	-0.031
	Sig.	0.918	0.633
	N	235	235
eGFR (mL/min/1.73 m²)	Correlation Coefficient	-0.303	-0.315
	Sig.	**2.0E-06**	**7.65E-07**
	N	237	237

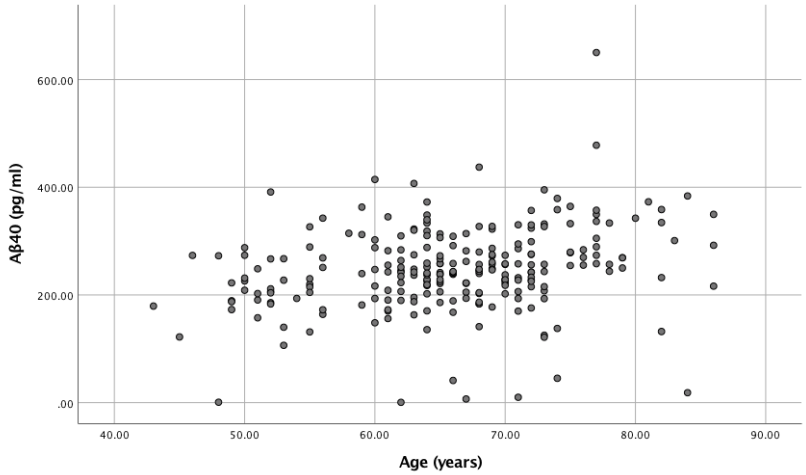

Figure 2-74: Scatter plot of age with β-amyloid 40 – SUMMIT no T2DM cohort. n=243, r=0.281, p=8.0E-6.

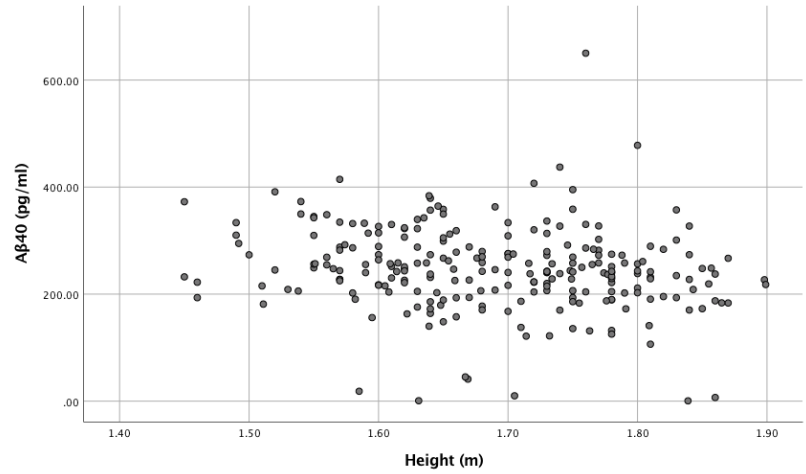

Figure 2-75: Scatter plot of height with β-amyloid 40 – SUMMIT no T2DM cohort. n=244, r=-0.222, p=4.8E-4.

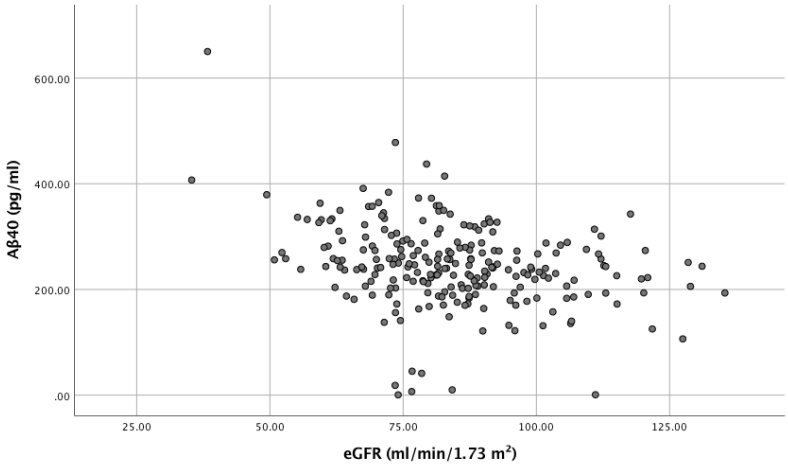

Figure 2-76: Scatter plot of eGFR with β-amyloid 40 – SUMMIT no T2DM cohort. n=237, r=-0.303, p=2.0E-6.

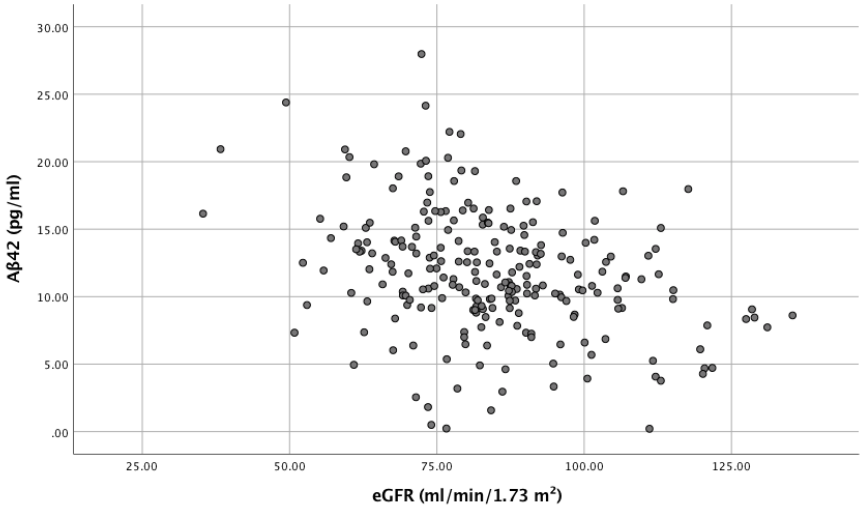

Figure 2-77: Scatterplot of eGFR with β-amyloid 42 – SUMMIT no T2DM cohort. n=237, r=-0.315, p=7.65E-7.

The above analysis looked at univariate correlations between plasma β-amyloid and continuous clinical determinants in SUMMIT subjects without T2DM. After adjusting for multiple comparisons, a significant positive correlation was seen between β-amyloid 40 and age, while significant negative correlations were present between β-amyloid 40 and height as well as eGFR. After the Bonferroni correction, the only significant association of β-amyloid 42 was a negative correlation with eGFR.

2.5.10 Association of Plasma β-amyloid with Binary Determinants: SUMMIT No T2DM Cohort

To analyse the association of different pharmacological agents, gender and CVD status with plasma β-amyloid in the non-diabetic cohort, new plasma β-amyloid quartiles were calculated using only the values of non-diabetic subjects. To adjust for multiple comparisons, the Bonferroni method set the new threshold for significance at $p<0.003$.

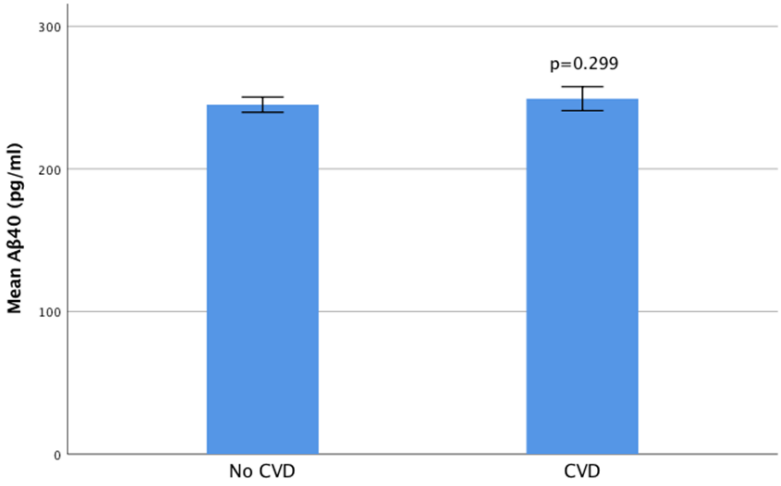

Figure 2-78: SUMMIT Non-T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with CVD, n=120, n=124 respectively.

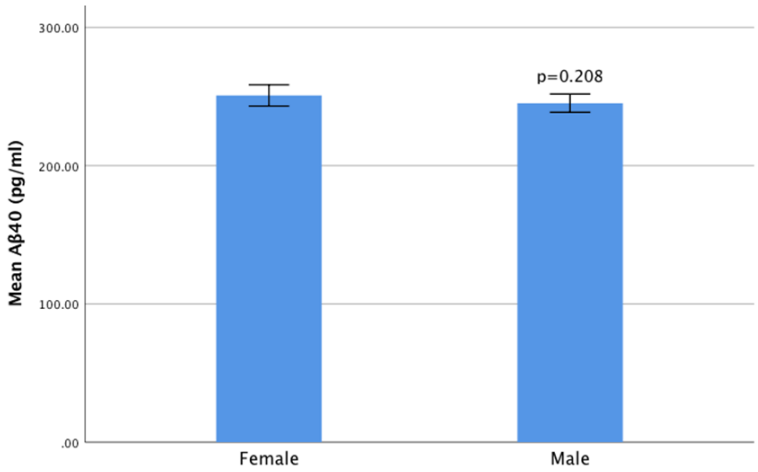

Figure 2-79: SUMMIT Non-T2DM cohort - comparison of β-amyloid 40 levels in females and males, n=96, n=147 respectively.

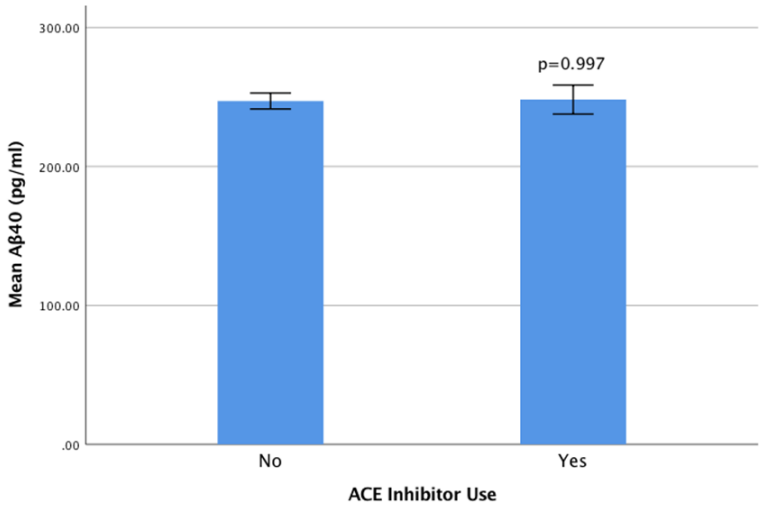

Figure 2-80: SUMMIT Non-T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with ACE inhibitor use, n=180, n=62 respectively.

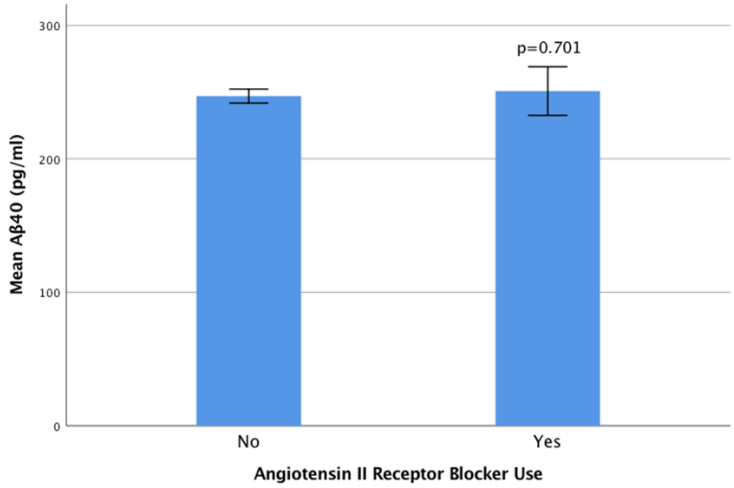

Figure 2-81: SUMMIT Non-T2DM cohort - comparison of β-amyloid 40 in subjects without and with angiotensin II receptor blocker use, n=218, n=24 respectively.

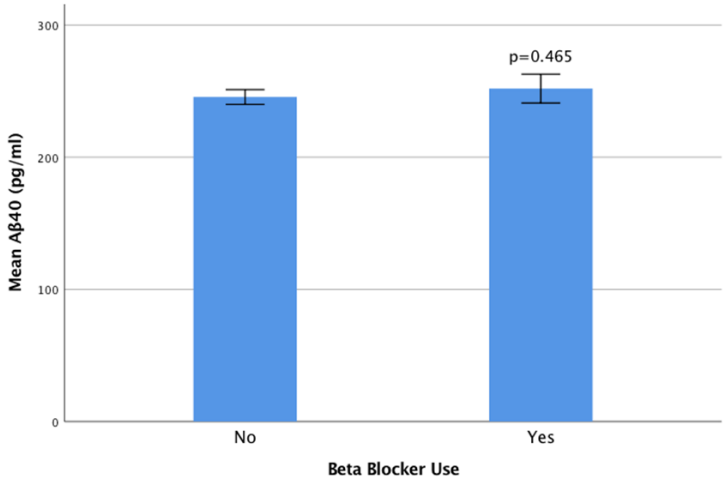

Figure 2-82: SUMMIT Non-T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with β-blocker use, n=178, n=65 respectively.

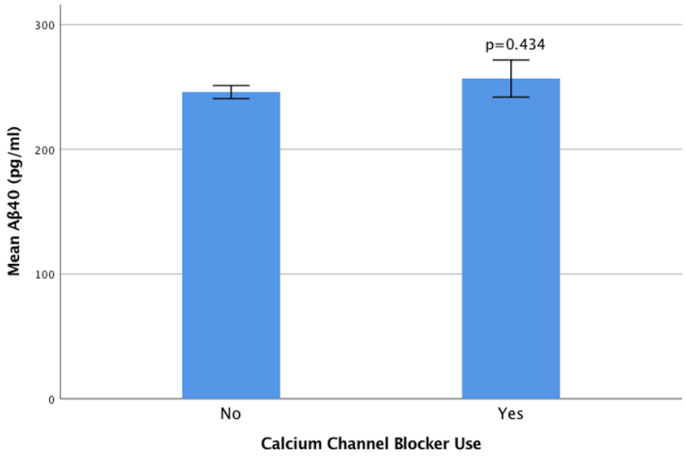

Figure 2-83: SUMMIT Non-T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with calcium channel blocker use, n=199, n=43 respectively.

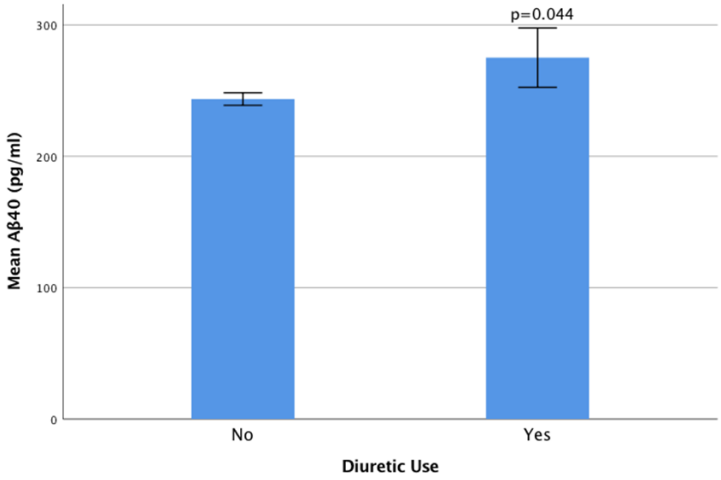

Figure 2-84: SUMMIT Non-T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with diuretic use, n=213, n=29 respectively.

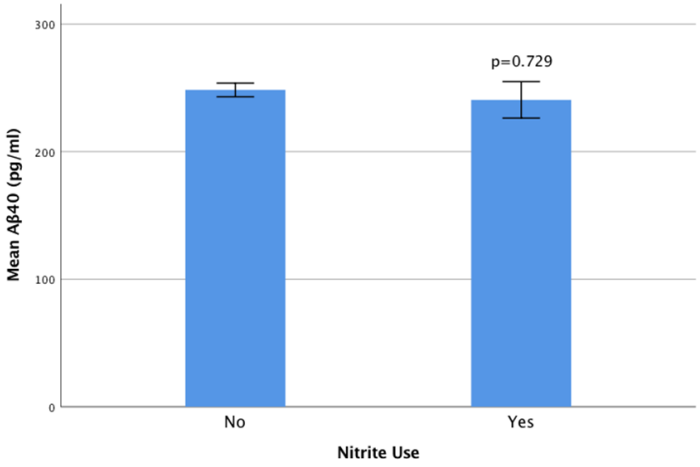

Figure 2-85: SUMMIT Non-T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with nitrite use, n=208, n=35 respectively.

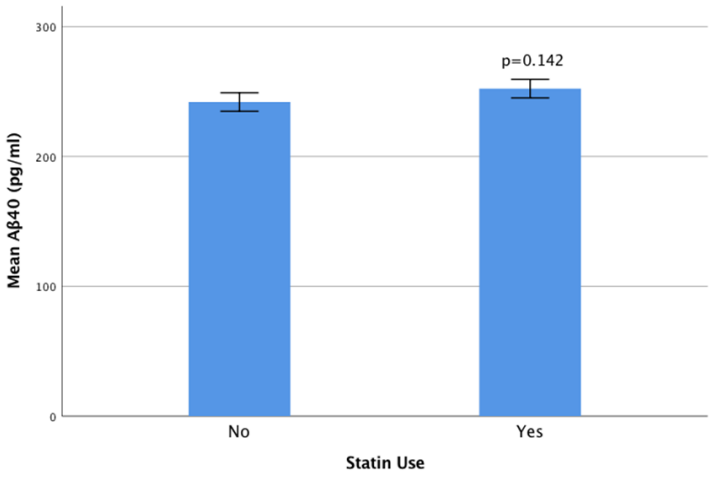

Figure 2-86: SUMMIT Non-T2DM cohort - comparison of β-amyloid 40 levels in subjects without and with statin use, n=113, n=128 respectively.

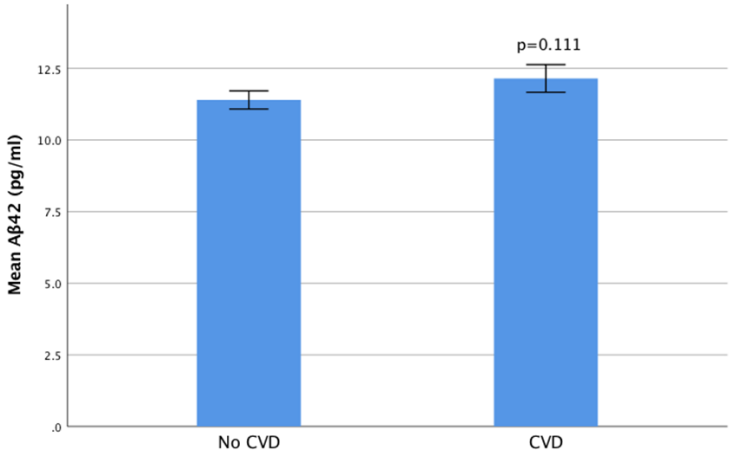

Figure 2-87: SUMMIT Non-T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with CVD, n=120, n=124 respectively.

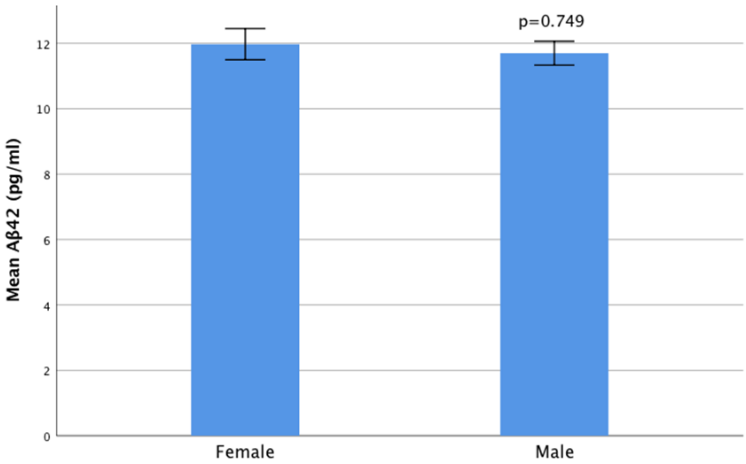

Figure 2-88: SUMMIT Non-T2DM cohort - comparison of β-amyloid 42 levels in female and male subjects, n=97, n=146 respectively.

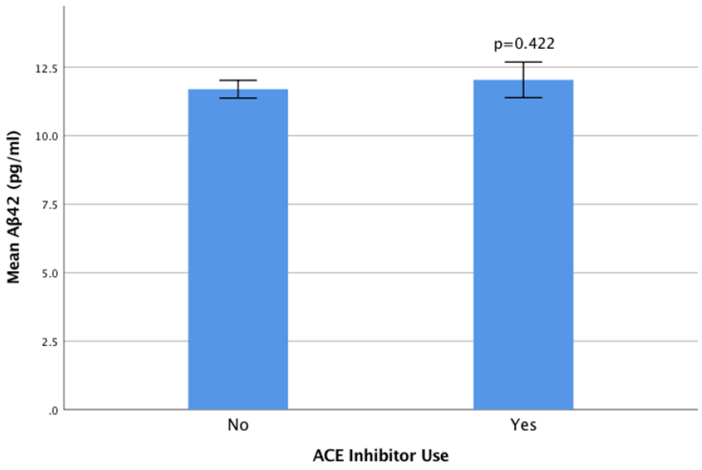

Figure 2-89: SUMMIT Non-T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with ACE inhibitor use, n=180, n=62 respectively.

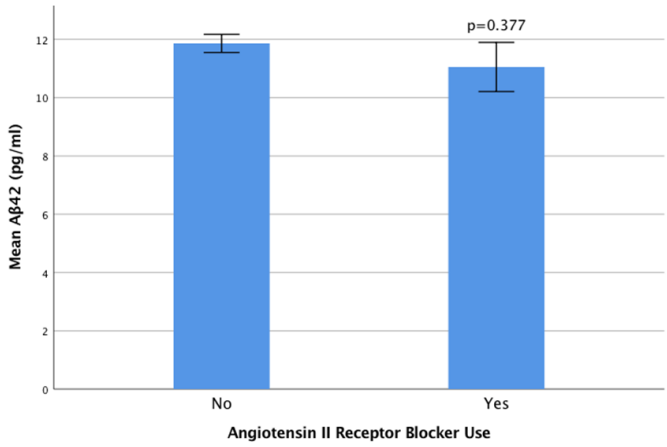

Figure 2-90: SUMMIT Non-T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with angiotensin II receptor blocker use, n=219, n=23 respectively.

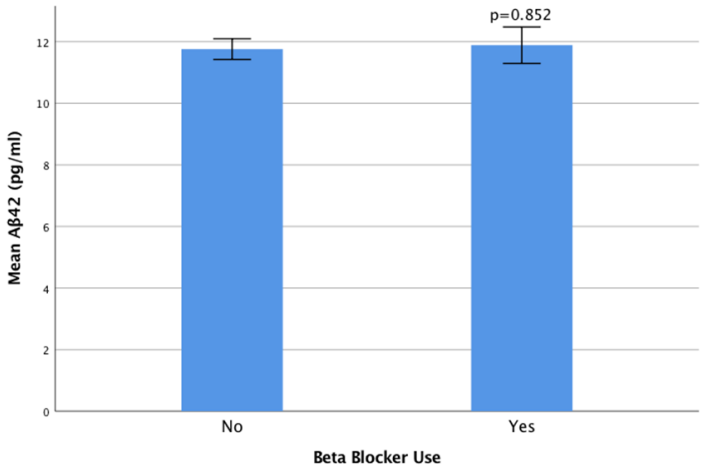

Figure 2-91: SUMMIT Non-T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with β-blocker use, n=179, n=64 respectively.

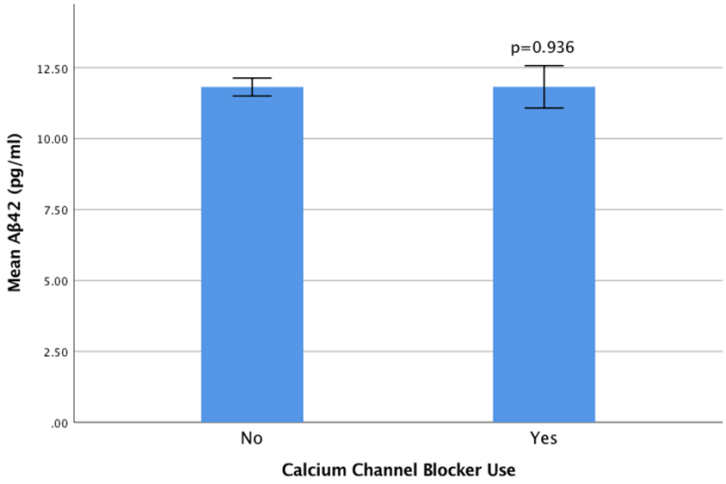

Figure 2-92: SUMMIT Non-T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with calcium channel blocker use, n=199, n=43 respectively.

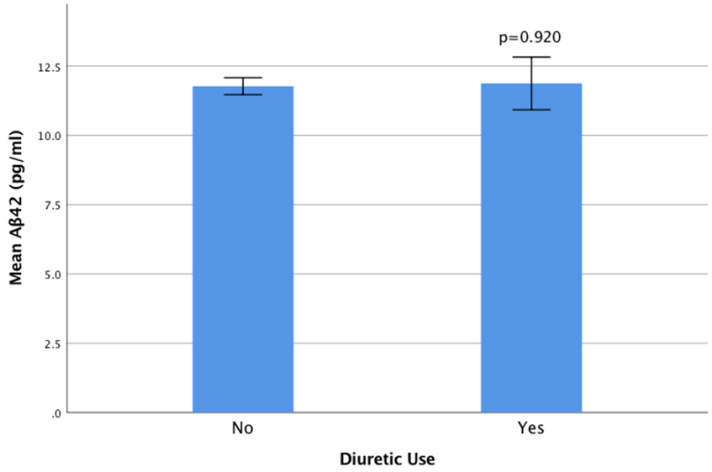

Figure 2-93: SUMMIT Non-T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with diuretic use, n=213, n=29 respectively.

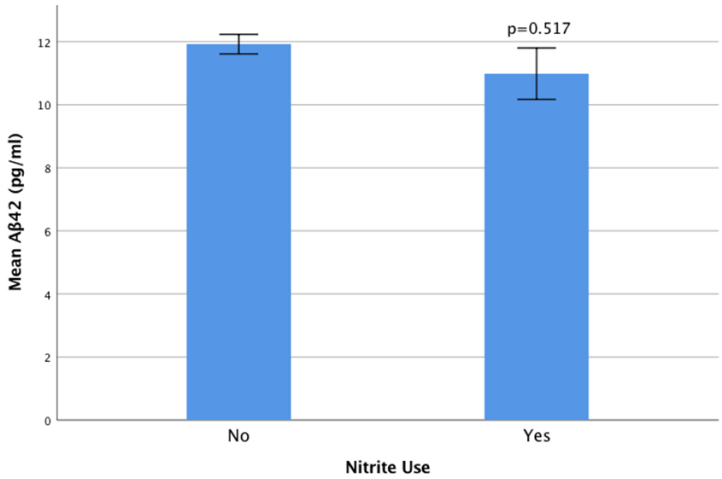

Figure 2-94: SUMMIT Non-T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with nitrite use, n=209, n=34 respectively.

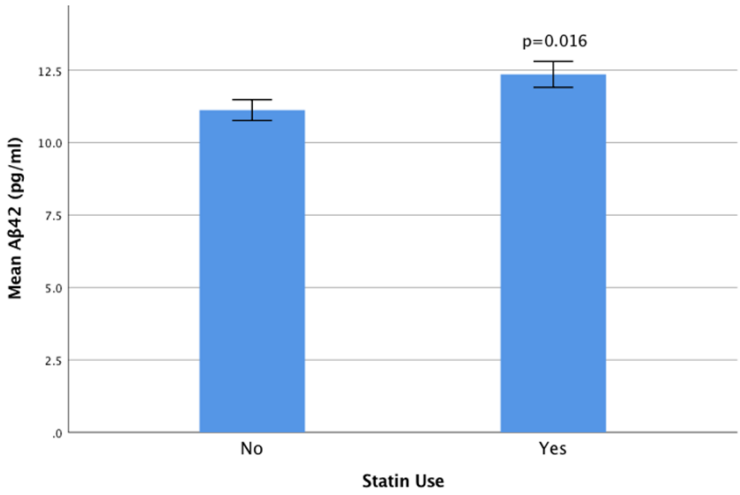

Figure 2-95: SUMMIT Non-T2DM cohort - comparison of β-amyloid 42 levels in subjects without and with statin use, n=113, n=128 respectively.

The above analysis looked at potential binary determinants of plasma β-amyloid in SUMMIT subjects without T2DM. After setting the new threshold for significance at $p<0.003$, no significant differences were found.

2.5.11 Regression Analysis of Independent Determinants of β-amyloid – SUMMIT No T2DM Cohort

In order to determine which factors are independent predictors of plasma β-amyloid in subjects without T2DM, a linear regression model was used. Significant variables identified from the univariate analyses above were entered into the model and selected using forward selection. As previously, the standard level of significance $p<0.05$ was used to select factors to be included in the regression. B refers to the unstandardized regression coefficient which represents the slope of the model associated with a 1 unit change in the independent variable. Beta refers to the standardised regression coefficient and allows for direct comparison of the effects of independent variables.

Table 2-9: Linear regression model with independent variables Age, BMI, Height and eGFR selected using forward selection.

β-amyloid 40			
Independent Variables	B	Beta	Sig.
eGFR (mL/min/1.73 m²)	-1.205	-0.263	3.00E-05
Age (years)	1.68	0.187	0.003
Height (m)	-123.937	-0.158	0.01

Table 2-10: Linear regression model with independent variables Weight, LDL cholesterol, eGFR, Statin use selected using forward selection

β-amyloid 42			
Independent Variables	B	Beta	Sig.
eGFR (mL/min/1.73 m²)	-0.095	-0.345	1.46E-07

As can be seen from the above regression analysis, eGFR, age and height were the only significant independent associations with β-amyloid 40. eGFR was the only independent significant factor to associate with β-amyloid 42.

2.6 Discussion:

The development of assays capable of measuring plasma β-amyloid was followed by excitement surrounding its potential use as a biomarker of AD. Disappointingly however, several studies have reliably shown that plasma β-amyloid is a poor predictor of the development or progression of AD (63). The above analysis perhaps sheds some light on the reasons behind its failure as a biomarker in this condition, as it suggests that plasma β-amyloid is potentially affected by a large number of patient characteristics and is also influenced by pharmacological agents. This study is the first of its kind to extensively look at determinants of plasma β-amyloid 40 and 42 in non-elderly subjects.

2.6.1 Plasma β-amyloid and Renal Function

The most significant association of plasma β-amyloid is estimated glomerular filtration rate, a surrogate marker of renal filtration function calculated on the basis of creatinine concentrations, a molecule filtered but poorly secreted and poorly absorbed by renal tubular cells. A strong inverse relationship between plasma β-amyloid and renal function has previously been reported by a number of studies (56,64). However, several gaps in our knowledge remain. Firstly, it remains to be determined whether free circulating β-amyloid is primarily renally excreted and therefore found to accumulate in subjects with declining renal function, or whether increased free circulating β-amyloid is nephrotoxic and results in a decline in renal function. If declining renal function is found to cause accumulation of free circulating plasma β-amyloid the possible causal mechanisms are plentiful. Being only 4kDa in size, it is possible that free circulating β-amyloid is simply filtered at the glomerulus and excreted in the urine. Indeed, despite the paradigm of a healthy glomerulus being impermeable to protein, it is now recognised that the filtration of smaller peptides as well as larger proteins at the glomerulus occurs even in the healthy state and is dictated primarily by peptide charge and size as well as glomerular slit size (65,66).

Another possible explanation takes into account other important processes carried out at nephron level including metabolism of various substrates. It is well known that

insulin is degraded at least in part by the kidneys (67,68). Therefore, it is possible that should β-amyloid prove to be a biomarker of CVD, it could simply be acting as a biomarker of renal function, with poor renal function being the underlying causal association

2.6.2 Plasma β-amyloid and Pharmacological Agents

As reviewed previously in the introduction, a small number of studies looking at the association between plasma β-amyloid and pharmacological agents exist. The findings in the present study reproduce some of the results presented previously. In agreement with previous findings, higher plasma β-amyloid 42 levels were significantly associated with insulin use in subjects with type 2 diabetes. A number of studies have previously reported an association between β-amyloid and insulin. Plasma β-amyloid was shown to correlate with levels of endogenous insulin (69). Additionally, administration of exogenous insulin was also shown to increase levels of circulating β-amyloid 42 (70). However, the reasons behind this association remain poorly understood. When looking at univariate analyses, higher plasma β-amyloid levels were consistently associated with presence of T2DM. Therefore, it is possible that the association between insulin use and plasma β-amyloid is simply a reflection of a longer duration of T2DM.

No significant relationship was found with plasma β-amyloid and NSAIDs or statins. An interesting finding is that in univariate analyses, plasma β-amyloid levels were significantly higher in subjects on angiotensin II receptor blockers but not ACE inhibitors. However, use of ARBs was not found to be independently associated with plasma β-amyloid levels in regression analyses.

Another interesting observation that remained statistically significant even in regression analysis, is the association of plasma β-amyloid with diuretic use. In both the SUMMIT cohort and the SUMMIT T2DM cohort, the use of diuretics was associated with an approximately 30pg/ml increase in plasma β-amyloid 40 levels. Unfortunately, information about use of specific types of diuretics was lacking in the database. Given that different types of diuretics function in different ways it is therefore difficult to try and speculate about potential mechanisms behind this

association. However, generic properties of most diuretics include inducing diuresis by means of natriuresis, with variable effects on other electrolytes. Although diuretics have been in widespread clinical use for decades and their molecular effect on the nephron is well understood, the effect of diuretics on eGFR is unknown. A small number of human studies have yielded conflicting results. Loon et al found that administration of furosemide to 9 hypertensive subjects with normal renal function resulted in a non-significant increase in eGFR, while Gottlieb et al found a decrease in eGFR of 12 subjects with congestive heart failure after administration of furosemide (71,72). Trivedi also concluded that acutely, administration of Furosemide results in a reduction in eGFR (73). Therefore, it is difficult to determine whether an increase in plasma β-amyloid in subjects on diuretics is due to their effects on eGFR.

2.6.3 Outliers in plasma β-amyloid and other clinical variables

As is evident from the preceding analysis, a number of outliers can be seen when looking at levels of plasma β-amyloid 40 and 42 as well as other baseline clinical parameters. 21 subjects were found to have low plasma β-amyloid 40 levels while 19 subjects were found to have low β-amyloid 42 levels. In an attempt to determine the reason behind this observation, we considered a number of different possible explanations. Firstly, we looked at the possibility of assay failure. However, the samples corresponding to low values of plasma β-amyloid 40 and 42 were scattered across a number of different assay plates and therefore assay failure was deemed unlikely. Another potential explanation is that a qualitative issue with the sample resulted in the degradation of our peptide of interest. However, all samples were stored under the same conditions and no significant abnormalities were seen when looking at other baseline biological plasma markers measured in the same sample. Additionally, we also found that although the values reported for some subjects were extremely low, they were still well within the minimum detection limit for the assay used to measure plasma β-amyloid concentrations. An interesting observation is that all of the samples with low levels of plasma β-amyloid are from subjects within the Exeter cohort. Therefore, it is possible that unknown biological and

genetic factors are responsible for this phenomenon. However, these cannot be reliably adjusted for. A possible solution in the future would be to perform an analysis of subjects recruited in the Exeter and Dundee cohorts separately, in an attempt to take into account unknown confounders and differences among the groups. This would also allow one to take into account the effects of different medication prescribing behaviours in different geographical areas within the UK. Other outliers were identified when looking at the baseline parameters of eGFR as well as LDL cholesterol. However, no clear explanation for such low values could be determined based on the data available. Therefore, all of the outliers discussed above were included in the analysis.

2.6.4 Limitations of Study

There are a number of limitations of this exploration of factors affecting plasma β-amyloid levels. Firstly, due to limited amounts of blood samples from subjects enrolled in the study, plasma β-amyloid was only measured as a one-off measurement in all of the samples. Therefore, any assay faults or poor quality of individual specimens cannot be accounted for. Another limitation directly related to only analysis one-off measurements of plasma β-amyloid is the fact that the effects of circadian variation cannot be accounted for. As with a number of circulating molecules, the natural circadian rhythm often exerts a considerable effect on their plasma concentrations. The most extreme example of this would be cortisol, where diurnal variations in plasma concentrations result in as much as a 50% increase in its concentrations shortly after awakening (74). Indeed, the results of one small study would suggest that plasma β-amyloid levels demonstrate variation based on circadian rhythms and that the amplitude of these variations diminishes with age (75). In the SUMMIT database, attempts were made at adjusting for circadian variation by wherever possible, recording measurements and collecting blood samples in the morning.

Yet another factor that could hinder attempts at investigating associations with circulating β-amyloid, is that in healthy humans, approximately 70% of β-amyloid 40

and 90% of β-amyloid 42 circulates in the bound form (76). Assays used to measure plasma β-amyloid measure only the free circulating form. As with other protein bound plasma molecules, changing between protein bound and free circulating forms is a dynamic process. Currently, factors affecting transitioning between protein bound and free circulating β-amyloid are unknown.

In terms of the analysis of pharmacological agents with plasma β-amyloid concentrations, a major limitation is the lack of information about specific medication types. Although the database provides information about the use of broad categories of medications such as diuretics, statins or NSAIDs, information about specific preparations is lacking. This is potentially significant, as a previous study found differing effects of different NSAID preparations on plasma β-amyloid levels. Additionally, given the strong association between diuretic use and plasma β-amyloid levels, a possible mechanistic explanation for this cannot be provided. This is because different classes of diuretics function via a number of different mechanisms.

Another major limitation of this study is the relatively imbalanced size of subgroups. There is a larger number of subjects in the diabetic cohorts compared to the non-diabetic cohorts. When performing an analysis of the baseline cohort, this may therefore skew the results towards a more diabetic phenotype. Indeed, it would seem that on a number of occasions, results seen in the baseline cohort are more closely related to those observed in the diabetic cohort. However, regression analysis of the SUMMIT cohort adjusted for diabetes status, therefore, this would likely resolve the issue of imbalanced groups. Another potential explanation for this observation is statistical power. Given that the non-diabetic subgroup will be statistically underpowered when compared to the diabetic subgroup, it is possible that the number of subjects simply diminishes the significance of any associations. The pre-existing database used in this study was not one specifically designed for the purpose of the above analyses, and therefore it was impossible to attempt to control factors such as adequate sample size.

2.6.5 Future Directions

Despite a large number of drawbacks, this study provides valuable, novel information about the factors affecting plasma β-amyloid concentrations and highlights a number of interesting associations that could be the subject of future research. Firstly, it would be of great interest to look at plasma β-amyloid levels in relation to some of the above factors in a new, purpose-designed study which would allow one to account for factors such as circadian variability. Additionally, further studies aiming to tease apart the mechanisms explaining the association between plasma β-amyloid and use of diuretic/ renal function would be of potential therapeutic significance for a range of β-amyloid associated pathological states.

3 Association of plasma β-amyloid with biomarkers of cardiovascular health and cardiovascular outcomes

3.1 Assessing Vascular Health in the Research Setting

As discussed in chapter 1, biomarkers of CVD currently in use have a number of limitations and struggle to identify a significant number of subjects at risk of major cardiovascular events. Therefore, the search for potential novel biomarkers continues to be important. Ideally, any novel biomarkers should be directly involved in the pathophysiological pathways in the disease of interest or should provide an accurate representation of the extent or severity of the pre-clinical stages of the disease. In the context of CVD, a number of methods are commonly used in the research setting to assess either structural or functional components of cardiovascular health and therefore provide a means of quantifying the extent or severity of pre-clinical stages of CVD. Therefore, by demonstrating an association between any potential novel biomarkers of CVD and pre-clinical functional/ structural changes in the vasculature, one can not only prove the usefulness of a novel biomarker in estimating pre-clinical disease extent, but hopefully also shed some light on the potential mechanisms behind any associations. Although plentiful methods for assessing vascular health in the research setting exist (see Table 3.1), the following review will focus on describing methods used in the dataset of interest.

Table 3-1: Summary of methods used for functional and structural assessment of the vascular tree in the research setting

Technique	Vascular Bed	Advantages	Disadvantages	Stimulus	CV Outcome Prediction
Venous occlusion plethysmography (77)	Microcirculation - Forearm circulation with brachial artery drug administration	Reproducible, contralateral limb as control, readily accessible vascular bed	Invasive, potential damage to brachial artery, lack of outcome studies	ACh, SNP, other vasoactive substances	No large outcome studies
Flow-mediated dilatation (78)	Brachial artery	Easily accessible vascular bed, gold standard with extensive outcome studies, relatively inexpensive	Operator dependent, extensive operator training required, poor standardisation	Reactive hyperaemia	(79)
Laser Doppler ± iontophoresis (80)	Skin microcirculation	Non-invasive, no observer dependency	Variability of skin perfusion, smaller evidence base	ACh, SNP, reactive hyperaemia	(81,82)
EndoPAT (83)	Digital microcirculation	Non-invasive, FDA approved, automated so not operator dependent	Expensive single-use probes	Reactive hyperaemia	(84,85)
Pulse wave analysis/velocity (86)	Global vascular assessment	Non-invasive, extensive evidence base for CVD outcomes	Uncertainty of true path length - obesity	NA	(87–89)
Ankle-brachial pressure index (90)	Global vascular assessment	Non-invasive, readily available in clinical areas	Observer dependency, less useful in subjects with calcification	NA	(91,92)
Carotid Intima-Media Thickness (93)	Common carotid artery	Reproducible, non-invasive, inexpensive	Lack of standardisation, some disagreement about correlation with coronary circulation	NA	(94,95)

3.1.1 Functional Assessment

Once considered only a simple layer of cells with little function, the endothelium is now recognised as an important regulator of vascular homeostasis. Indeed, endothelial dysfunction or activation in response to a range of different stimuli is thought to be the first step in the development of atherosclerosis, long preceding the onset of structural changes. The idea that atherosclerosis was not solely a structural problem was first introduced in 1986 by Ludmer et al. Using intracoronary infusions of acetylcholine (ACh) combined with quantitative angiography they demonstrated that atherosclerosis in the coronary arteries was as much a structural as it was a functional defect (96). Since then, several safer and less invasive methods have been developed to measure endothelial function and these have consistently shown that deranged endothelial function plays a crucial role in the pathogenesis of atherosclerosis.

3.1.1.1 *EndoPAT*

EndoPAT is a non-invasive device designed to record beat-beat changes in pulse wave amplitude in the digital circulation. A probe is placed on the finger, and baseline recordings are measured. A blood pressure cuff is then inflated to above systolic pressure in order to occlude the circulation to the hand. After 5 minutes, the cuff is released and changes in pulse wave amplitude are compared to baseline (83) (See Figure 3-1, diagram adapted from (97).

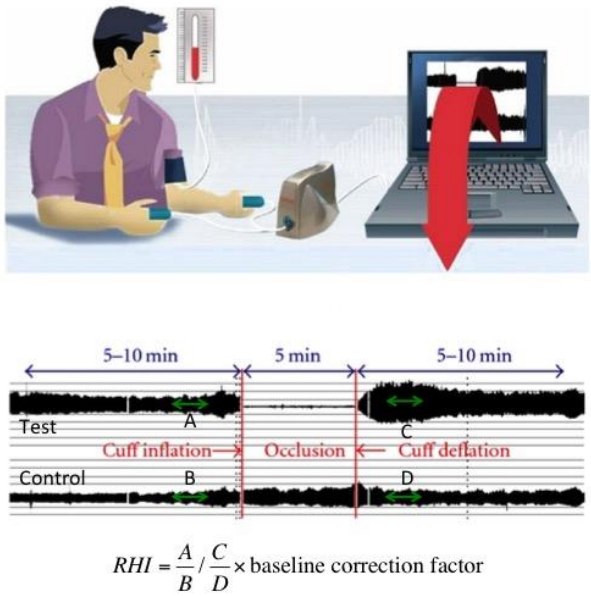

Figure 3-1: Diagram depicting set up for measurement of the reactive hyperaemia index (RHI) using the EndoPAT device. Diagram adapted from (97)

This method of measuring endothelial function has several advantages including its non-invasive nature, a fully automated procedure, the ability of the contralateral arm to serve as a control for any systemic changes in vascular tone as well as approval from the Food and Drug Administration (FDA), USA as a measure of endothelial function. However, a major limitation is the cost of the single-use finger probes. Critics of this method also highlight, that the digital circulation is perhaps not the most useful vascular bed for measuring endothelial function, as it is highly sensitive to a range of different factors including temperature and state of the autonomic nervous system (98).

3.1.1.2 *Laser Doppler and Skin Microcirculation*

Another method used to functionally assess the endothelium is the use of laser Doppler on the skin microcirculation in combination with iontophoresis of vasoactive drugs, reactive hyperaemia or heating. This form of vascular function assessment takes advantage of the Doppler effect, whereby light via a low power laser

illuminates the skin and undergoes a shift in frequency proportional to the velocity of red blood cells within the skin vasculature. (See Figure 3-2)

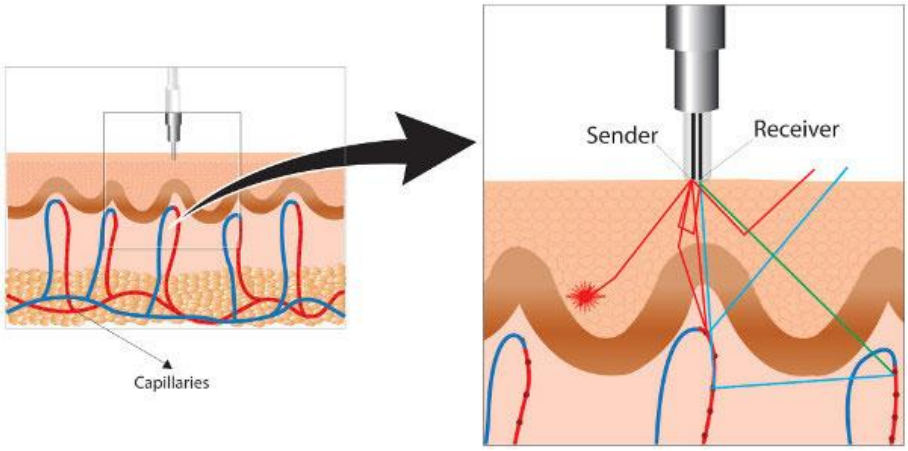

Figure 3-2: Using the Doppler effect to measure endothelial function in the skin microcirculation. A helium-neon laser can penetrate skin layers through to the dermis up to a depth of approximately 1.5mm. Image from (99)

Originally, 2 methods utilising this effect were available. Laser Doppler flowmetry analyses blood flow in a small area but with a high sampling frequency. It therefore has very good temporal resolution but poor spatial resolution, which, given the heterogeneity in skin perfusion is a potential limitation and accounts for poor reproducibility as a one-off measurement of baseline (80). Laser Doppler imaging on the contrary provides better spatial resolution, but has a low sampling frequency and is therefore relatively insensitive to rapid changes in skin perfusion (80). However, laser Doppler imaging can be combined with iontophoresis of vasoactive substances such as ACh, whereby a small a current (µA) drives charged molecules of vasoactive drugs across the skin. Whilst it is not the method of choice for assessing dynamic changes in blood flow, with serial measurements at different doses this set up allows for the generation of a dose response curve and thus quantifies the ability of the endothelium to respond adequately to a given stimulus. To further increase the speed of image acquisition, several groups have optimised the protocol with changes such as reducing the size of the area to be scanned or increasing the scanning speed (80). A further advantage of this method is that depending on the vasoactive substance applied, either endothelium dependent or independent

vasodilation can be assessed. When ACh is used it binds to M3 muscarinic receptors on endothelial cells and leads, in part, to the endothelial production of nitric oxide (NO) (in addition prostacyclin and endothelium-derived hyperpolarising factor) thus measuring an endothelium-dependent form of vasodilation. Sodium nitroprusside (SNP) on the other hand acts as a direct NO donor, and thus by-passes the endothelial component acting directly on vascular smooth muscle cells (VSMC). Other stimuli that can be applied to the vascular bed and have their response measured using this method include vascular perfusion response to a heat stimulus or reactive hyperaemia following temporary occlusion of blood supply distally (80).

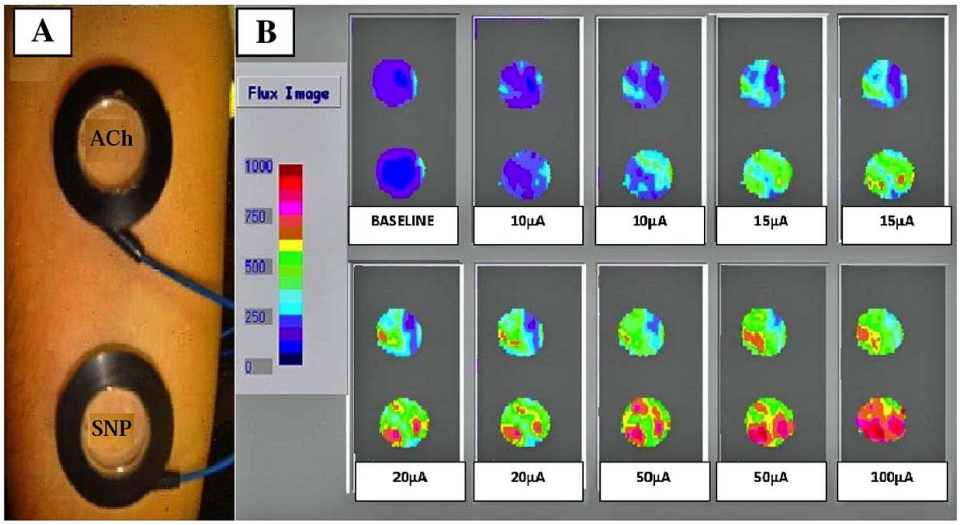

Figure 3-3: (A, B) Iontophoresis chambers used for drug delivery on subject's forearm. The polarity of chambers can be altered depending on the charge (positive or negative) of drug in solution. Image from (80)

To combat the limitations of both laser Doppler imaging and laser Doppler flowmetry, a newer technology, laser speckle contrast imaging has been developed which provides both good spatial and temporal resolution.

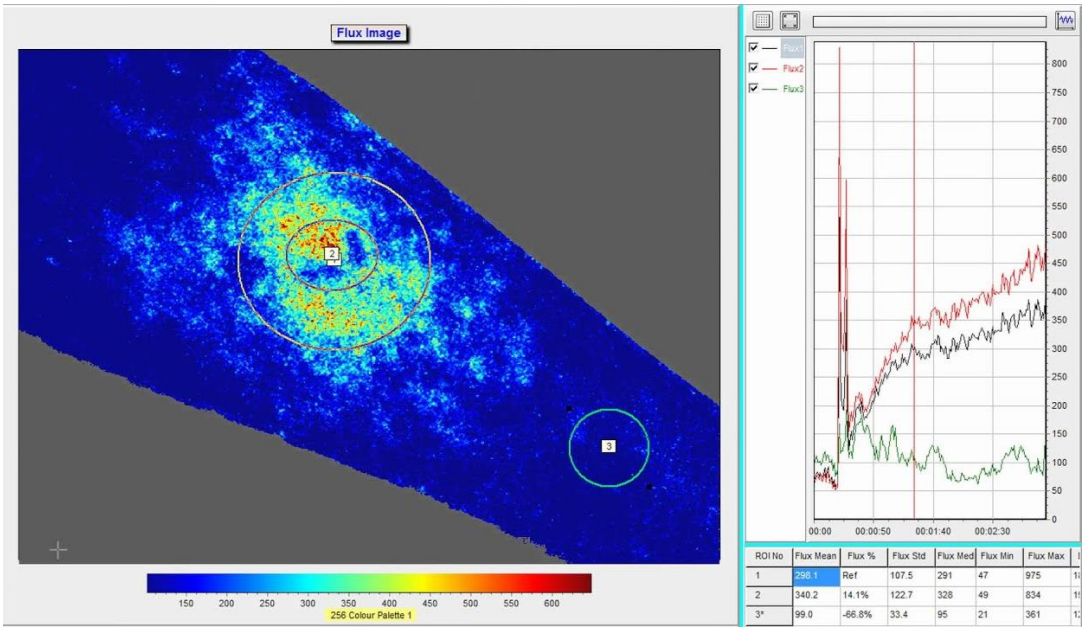

Figure 3-4: Image obtained using the Laser speckle contrast imager by Moor Instruments (100)

Clearly, this field is of great interest as the use of the skin microcirculation as a surrogate marker of general vascular health provides many advantages. Not only is it an easily accessible and non-invasive method of assessment, but studies have shown a significant positive correlation between skin microvascular function assessed by means of laser Doppler imaging and ACh/SNP iontophoresis and measures of coronary vascular function (101).

3.1.2 Structural Assessment:

Functional assessment of the endothelium allows detection of early, pre-clinical disease prior to the formation of structural changes. As such, these techniques are less useful for measuring atherosclerotic disease burden in established atherosclerosis. Several methods looking at vascular structure as opposed to function have been developed for this purpose.

3.1.2.1 *Carotid Intima-Media Thickness*

One commonly used structural arterial test is measurement of carotid intima media thickness (IMT). The common carotid (CC) arteries terminate at the carotid bulb, which divides to form the internal and external carotid artery (see figure 3.5 - images from (102,103).

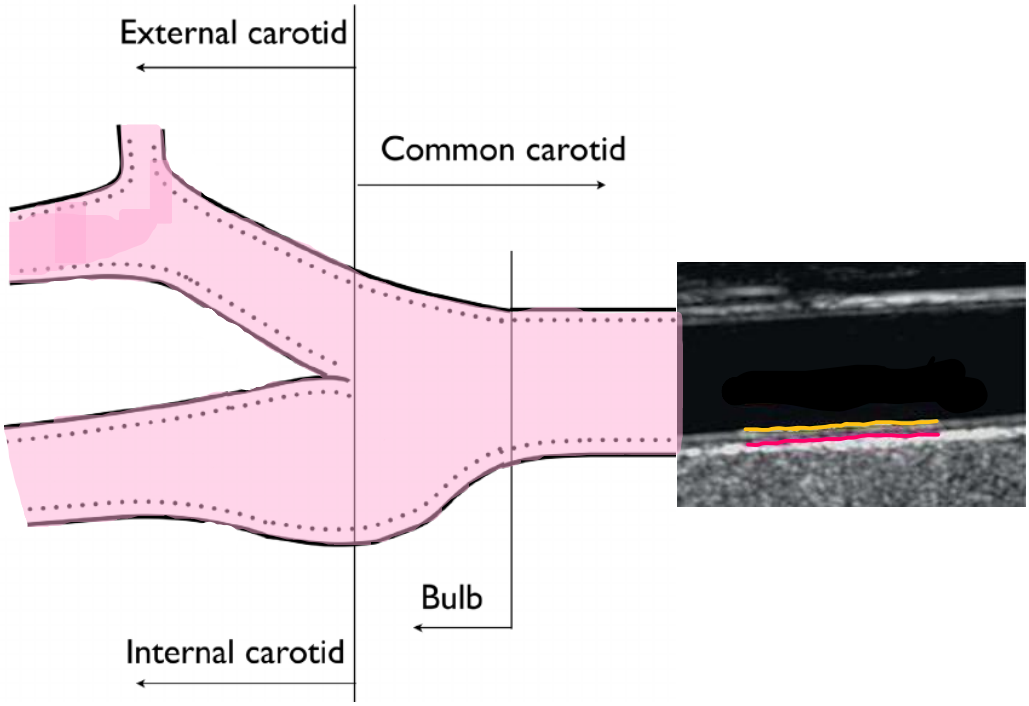

Figure 3-5: Carotid intima media thickness is measured as the distance between the lumen-intima boundary (in yellow) and the intima-media boundary (in pink). Figure from (102,103)

Using ultrasound, the distance between the intima-lumen interface and media-adventitia interface is measured in multiple segments along the course of the CC arteries. Studies have shown that a 0.2mm increase in carotid intima media thickness is associated with a 33% increase in risk of myocardial infarction and a 28% increase in risk of stroke (104). Additionally, on the contrary to commonly used cardiovascular risk scores, determining risk based on carotid intima thickness takes into account the effects of risk modifying agents such as statins. Indeed, a randomised meta-analysis of randomised controlled trials concluded that statin therapy was associated with a favourable decrease in CC artery IMT (105). Other

useful information that can be obtained using this method is assessment of plaque size and stability (105).

3.1.3 Assessment of Arterial Stiffness – Pulse Wave Velocity and Augmentation Index

The arterial tree has 2 distinct functions – a conduit function ensuring tissue perfusion and the cushioning functioning. This serves to transform a pulsatile flow generated by myocardial contraction into a continuous flow and also dampens down individual fluctuations in blood pressure. The ability of the arterial system to adequately perform these functions is in part dictated by arterial stiffness. Upon ventricular contraction, a forward pressure wave is generated which is reflected at different parts of the arterial tree and travels back as the reflected pressure wave. When arteries are compliant, the timing of the reflected pressure wave is such that it reaches the central arteries during late systole/early diastole, thus contributing to diastolic pressure (DBP) and coronary perfusion. However, when arterial stiffness increases, the velocity of the incident as well as reflected pressure wave increases and the reflected wave arrives back within central arteries at an earlier point in the cardiac cycle (107). It is precisely this mechanism that is responsible for the phenomenon of increased pulse pressure in elderly individuals, whereby an early, reflected wave joins the incident pressure wave causing an increased systolic pressure, but reduced DBP. The amount by which the early reflected pressure wave alters the normal central pressure waveform is measured as the augmentation index (AI) (see figure 3-6, diagram from (108)). Understandably, due to the normal physiology of coronary perfusion, preload and afterload, this phenomenon therefore results in reduced coronary perfusion and increased cardiac afterload.

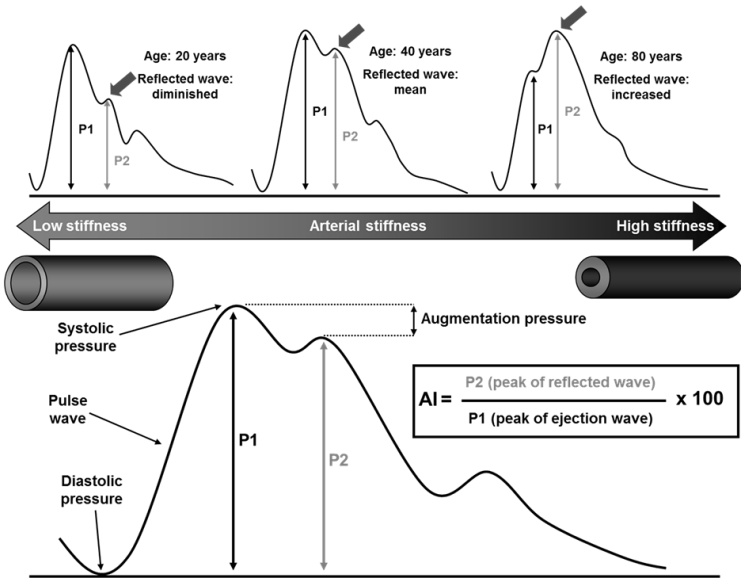

Figure 3-6: Diagram of pulse waveforms seen in central arteries of different stiffness. P1= peak of the incident wave. P2 = peak of the reflected wave. Figure from (108)

A commonly used device to measure pulse wave velocity is the SphygmoCor system. According to the manufacturer's protocol, the subject is to be placed supine and resting for at least 5 minutes. A thigh cuff is placed as far up the thigh as possible and the carotid pulse is then located on the neck. The cuff placed around the thigh records femoral waveform data whilst a hand-held tonometer records the waveform in the carotid artery (see figure 3-7, image from (109)). Distances between the cuff, sternal notch, femoral pulse and carotid pulse, allows the software to calculate the transit time from carotid to femoral artery to give the pulse wave velocity. Whilst radial:carotid transit times can also be measured, carotid to femoral measurements remain the gold standard (110).

Figure 3-7: SphygmoCor system for measuring arterial stiffness. Figure from (110)

Although a completely non-invasive and relatively fast method for measuring arterial stiffness, a major disadvantage is the requirement for extensive operator training, as obtaining good quality, reproducible waveform tracings for at least 10 seconds can be challenging. Another major challenge is that in addition to the SphygmoCor system described above, other devices for measuring pulse wave velocity exist, such as the Arteriograph and Complior systems. Studies have shown that these systems produce significantly different results and values obtained using different systems therefore cannot be directly compared (111). However, measurement of pulse wave velocity in the research setting is of value as it has been extensively validated and shown to predict cardiovascular outcomes in a number of large studies. A meta-analysis by Vlachopolous et al. looked at 17 longitudinal studies measuring aortic pulse wave velocity. They report a stepwise, linear increase in clinical events with increasing pulse wave velocity tertiles, but note that arterial stiffness was the strongest predictor of cardiovascular outcomes in individuals with a higher baseline risk (88). Among patients with diabetes, several studies have shown that arterial stiffness is related to the progression of cardiovascular complications, whilst other studies have shown that changes in arterial stiffness occur even in the pre-diabetic stages (112).

3.1.4 β-amyloid and cardiovascular disease

As mentioned in the previous chapter, β-amyloid is a peptide investigated primarily in the context of AD and CAA. Indeed, plasma β-amyloid was not one of the main biomarkers initially measured in the SUMMIT database, and was only measured retrospectively in a subset of the dataset on the background of interesting findings from a mouse study by Meakin et al. (33). By retrospectively measuring β-amyloid levels in samples from a pre-existing cardiovascular database, the current translational follow up MSc project allows for the investigation of previous findings in the human population. The following review will aim to summarise our current knowledge of β-amyloid in the context of CVD processes.

3.1.4.1 *Animal Studies*

Many early hypotheses suggested that AD was caused by atherosclerosis of the cerebral vasculature. While this hypothesis is no longer widely accepted, a link between, β-amyloid levels and vascular dysfunction persists with a considerable evidence base from experimental animal models. Van De Parre et al. used the atherosclerosis-prone ApoE knockout mice (ApoE -/-) and ApoE-/- APP-/- mice and found that after feeding with a high-fat diet, atherosclerotic plaque size was significantly reduced in thoracic and abdominal aorta regions in APP -/- mice. They also report that ApoE-/- APP-/- mice have greater plaque stability (113).
Other studies have shown that β-amyloid has direct vasoactive properties. One study looked at transgenic mice over-expressing APP, and found that following somatosensory stimulation, mice over-expressing APP had significantly smaller increases in cerebral blood flow. Additionally, impaired cortical blood flow was shown to correlate with β-amyloid levels in the brain and was also reproduced in wild type mice after topical administration of β-amyloid 40 (114). Other studies have shown that β-amyloid mediated changes in vasomotor tone occur in an endothelium-dependent manner. Dietrich et al. looked at adenosine triphosphate (ATP)-induced vessel constriction in rat arterioles after administration of β-amyloid. Both β-amyloid 40 and β-amyloid 42 were shown to result in a significant reduction in the vasodilator response to ATP administration (32). More recently, the role of β-amyloid has also been looked at in the systemic vasculature. Preliminary data from our group showed that BACE1 staining was increased in human temporal arteries with atherosclerosis. Additionally, increasing levels of β-amyloid in mice over a longer period of time via infusion of mouse β-amyloid caused a significant reduction in the vasodilator response of the skin microcirculation to ACh iontophoresis when compared to mice infused with a scrambled β-amyloid peptide. Treatment of high fat diet fed mice with the BACE1 inhibitor Merck-3 improved vascular responsiveness to ACh and decreased β-amyloid concentrations by approximately 50% (115).

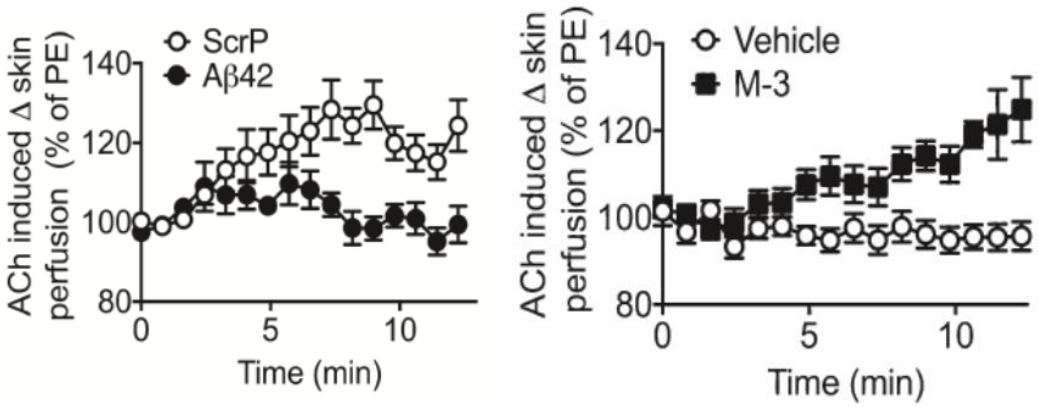

Figure 3-8: Unpublished results by Meakin et al – effect of β-amyloid on vascular response to ACh in mice. ACh- acetylcholine, ScrP-Scrambled peptide, M-3- BACE1 inhibitor Merck-3.

3.1.4.2 *β-amyloid and cardiovascular disease: Evidence from human studies*

Several studies have also looked at β-amyloid in relation to human vascular health in vivo. Beach et al. analysed the severity of atherosclerosis in the circle of Willis in 215 subjects with AD, 30 subjects with vascular dementia and 92 subjects without any form of dementia. Subjects with AD were found to have more advanced atherosclerosis than subjects in other groups (116). One small-scale study demonstrated a relationship between increased β-amyloid deposition within the brain and higher systolic blood pressure as well as pulse pressure in healthy, middle-aged individuals. Due to increased pulse pressure values, they postulated that this was likely due to changes in arterial stiffness (117). To follow this up, Hughes et al. used positron emission tomography scans to quantify β-amyloid deposition in the brains of 81 non-demented individuals and found that greater brachial-ankle pulse wave velocity was associated with higher levels of β-amyloid deposition in the brain at both baseline and 2 year follow up. However, carotid-femoral pulse wave velocity was not significantly increased in those with higher β-amyloid deposition in the brain (118).

Whilst understandably the majority of research thus far has focused on β-amyloid levels within the brain, some more recent studies have looked at link between circulating β-amyloid and vascular health. Stamatelopoulos et al. retrospectively measured β-amyloid 40 in blood samples from patients previously enrolled in other cardiovascular studies and found that circulating β-amyloid 40 was an independent

predictor of death and major adverse cardiac events in patients with coronary heart disease. After measuring pulse wave velocity at both baseline and at a median 4.4 years of follow up, increased β-amyloid 40 levels were also independently associated with progression of arterial stiffness (38).

3.2 Aims

Clearly, there is evidence that β-amyloid is a potential, but relatively unexplored risk factor in CVD. However, more research is required to tease apart this relationship and to better understand the role of other β-amyloid peptides, the relationship with diabetes as well as other markers of vascular health and pre-clinical atherosclerosis. The following analysis will aim explore the relationship between circulating β-amyloid and functional and structural biomarkers of cardiovascular health described above, as well as its association with CVD and outcomes. Additionally, this analysis will aim to determine whether any associations of plasma β-amyloid with biomarkers of cardiovascular health or cardiovascular outcome differs between subjects with or without diabetes.

3.3 Hypothesis

Based on evidence from our previous animal study, it is hypothesised that plasma β-amyloid will be primarily associated with functional vascular changes and that higher β-amyloid levels will be associated with impaired endothelium dependent functional responses to stimuli. Based on evidence from previous human studies reviewed above, it is also hypothesised that higher plasma β-amyloid 40 levels will associate with impaired arterial stiffness as well as impaired endothelium-independent functional responses to stimuli.

3.4 Methods

3.4.1 Study Population:

For a detailed description of the study population refer to chapter 2.

3.4.2 Structural and Functional Vascular Measurements

The following techniques for functional and structural vascular assessment had been previously used in the pre-existing SUMMIT database:

1. EndoPAT – Reactive Hyperaemia index
2. Arterial stiffness using the SphygmoCor device– pulse wave velocity
3. Ultrasound measurement of carotid intima-media thickness (cIMT) – left and right-sided common carotid IMT, left and right-sided common carotid bulb IMT
4. Laser Doppler Iontophoresis – Reactive hyperaemia peak response, SNP peak response, ACh peak response

More details about the exact methodology for each of the above vascular measurements can be found in the study by Shore et al. and Casanova et al (60,119). Due to technical problems related to low laser power and hence reduced sensitivity of accurately picking up lower perfusion values with the laser Doppler device at the Dundee centre, the first half of SNP and ACh dose response curves are not reliable and have as a consequence not been included for analysis purposes. Therefore, while the standard approach to analysing this data would be a comparison of repeated measures dose response curves and the corresponding regression model, the following analysis will only focus on the mean peak response to ACh or SNP, calculated by taking the mean average of the last 3 readings in each individual subject's dose response curve. This was done under the assumption of an accurately functioning laser Doppler device at higher perfusion values.

3.4.3 Statistical Analysis

All statistical analyses were performed using the SPSS statistical software package version 25. Distribution of data was analysed for normality using the Shapiro-Wilk test. Differences in clinical characteristics between groups were investigated using Chi-square, Kruskal-Wallis or Mann-Whitney U-tests, as appropriate. Where the distribution of both assessed variables was normal, Pearson correlation was used.

Where the distribution of one or both variables was not normal, correlations were investigated using Spearman's rho. A linear regression model was built using established cardiovascular risk factors from the Framingham and ASSIGN risk score calculators, β-amyloid, and significant determinants of β-amyloid identified in chapter 1. To analyse the association of plasma β-amyloid with clinically manifest CVD, the Chi-square test and Mantel-Haenszel linear by linear association test was used to explore the relationship between β-amyloid quartiles and CVD type at enrolment. To analyse whether β-amyloid was independently associated with follow up cardiovascular events, binary logistic regression was used. This analysis was only performed in the SUMMIT baseline cohort due to small numbers of accumulated outcomes.

3.4.4 Terminology

The following terms will be used throughout the analysis: the baseline SUMMIT cohort refers to the grouped cohort analysing both subjects with and without diabetes from both Dundee and Exeter centres of recruitment. The SUMMIT subjects with diabetes cohort will refer to subgroup analysis where only subjects with diabetes are included in the analysis (from both Dundee and Exeter). The same will apply for when the term SUMMIT subjects without diabetes cohort is used. The term Dundee baseline and Exeter baseline cohort will refer to all subjects from the corresponding centre of recruitment. Where the phrase 'any CHD' is used, this refers to all forms of coronary heart disease coded on the basis of previous myocardial infarction, unstable angina, stable angina, previous percutaneous coronary intervention (PCI) or previous coronary artery by-pass graft (CABG). Where the phrase 'any cerebrovascular disease' is used, this refers to all forms of cerebrovascular disease coded on the basis of stroke or transient ischaemic attack (TIA). Where the phrase 'any lower extremity arterial disease' (LEAD) is used, this refers to all forms of LEAD coded on the basis of intermittent claudication or previous diagnosis of LEAD.

3.5 Results

3.5.1 Patient Demographics

The following table of baseline characteristics the SUMMIT cohort has been displayed in chapter 2 but for ease of reading the details have been summarised below. Where the variable presented is a count, the number in brackets represents the equivalent %, where the number presented is a continuous variable, the number in brackets represents the standard deviation of that variable. Where significant differences exist between groups, the significance is illustrated using an asterisk (* = p<0.05, ** = p<0.01, *** = p<0.001).

Table 3-2: Characteristics of the SUMMIT baseline cohort.

SUMMIT Baseline Cohort				
	T2D with CVD	T2D no CVD	No T2D no CVD	No T2D with CVD
N	189	218	120	125
Male sex n (%) * **	142 (75%)	120 (55%)	53 (44%)	94 (75%)
Age years * **	67.7 (8.0)	63.9 (8.7)	63.1 (8.0)	68.5 (7.5)
T2D Duration years * **	12.0 (8.3)	8.6 (6.1)	NA	NA
BMI (kg/m2) * **	31.3 (5.1)	32.6 (5.9)	26.8 (4.3)	28.1 (4.1)
Medication				
Statin use * **	168 (89%)	162 (74%)	20 (17%)	108 (86%)
Antihypertensive use * **	169 (89%)	138 (63%)	18 (15%)	93 (74%)
Blood Pressure				
SBP	132.5 (18.1)	133.2 (16.3)	130.2 (16.4)	131.5 (18.3)
DBP * **	73.1 (8.2)	77.3 (8.8)	77.1 (9.0)	74.8 (8.9)
Metabolic parameters				
HbA1c mmol/mol * **	61.9 (15.6)	59.0 (14.8)	40.1 (4.1)	39.0 (3.3)
Total Cholesterol mmol/l * **	3.8 (0.9)	4.1 (0.9)	5.4 (1.0)	4.2 (0.9)
LDL Cholesterol mmol/l * **	1.8 (0.7)	2.0 (0.8)	3.1 (0.9)	2.2 (0.8)
HDL Cholesterol mmol/l * **	1.2 (0.3)	1.3 (0.4)	1.6 (0.4)	1.4 (0.4)
Triglycerides mmol/l * **	1.8 (1.1)	1.8 (1.0)	1.4 (0.8)	1.3 (0.7)
Renal Function				
Serum Creatinine umol/l * **	92.6 (33.2)	78.0 (20.3)	74.3 (13.7)	84.0 (19.4)

ACR mg/mmol ***	6.9 (34.3)	2.7 (5.5)	0.9 (1.1)	1.9 (4.3)
eGFR mL/min/1.73 m² ***	77.2 (22.3)	86.1 (22.6)	86.9 (16.2)	82.0 (17.5)

3.5.2 Analysis of the SUMMIT Baseline Cohort

3.5.2.1 *Correlation of Plasma β-amyloid with Markers of Cardiovascular Health*

In order to determine whether an association exists between plasma β-amyloid and markers of cardiovascular health, the first step was to use univariate correlations. Pearson or Spearman correlations were used as appropriate depending on the distribution of variables. The Bonferroni method was used to adjust for multiple comparisons, with the new threshold for statistical significance set at $p<0.003$. Simple scatter plots for significant correlations are displayed below.

Table 3-3: Univariate correlations of plasma β-amyloid with markers of structural and functional change.

Vascular Measurement	Correlation	Aβ40	Aβ42
Mean Peak SNP Response	Coefficient	-0.249	-0.003
	Sig.	**3.01E-09**	0.942
	N	553	553
Mean Peak ACh Response	Coefficient	-0.211	-0.40
	Sig.	**5.31E-07**	0.350
	N	555	555
Reactive Hyperaemia Index (EndoPAT)	Coefficient	-0.044	-0.051
	Sig.	0.275	0.207
	N	605	605
Reactive Hyperaemia Peak Perfusion	Coefficient	0.085	-0.13
	Sig.	0.063	0.004
	N	480	481
Pulse wave velocity	Coefficient	0.282	0.114
	Sig.	**2.38E-11**	0.008
	N	540	541
Mean common carotid IMT Right	Coefficient	0.14	0.077
	Sig.	**4.60E-04**	0.055
	N	619	618
Mean bulb IMT Right	Coefficient	0.042	-0.033

	Sig.	0.354	0.468
	N	499	499
Mean common carotid artery IMT Left	Coefficient	0.107	-0.001
	Sig.	0.007	0.971
	N	621	621
Mean bulb IMT Left	Coefficient	0.109	0.024
	Sig.	0.012	0.579
	N	523	524

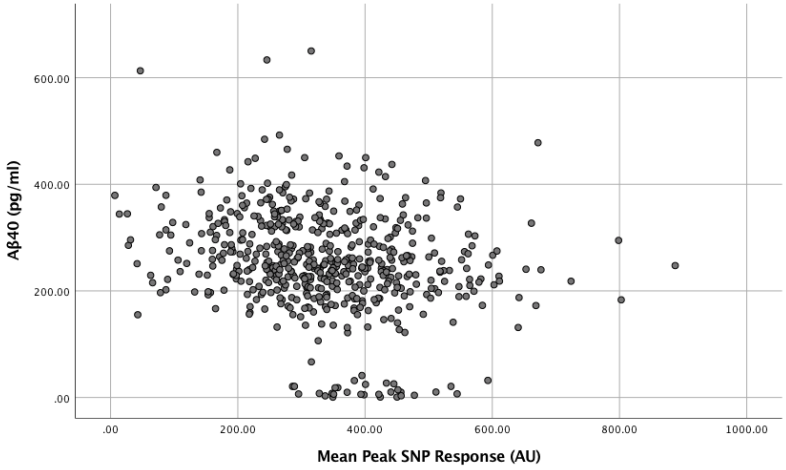

Figure 3-9: Scatter plot of plasma β-amyloid 40 with the average of the 3 last readings on the SNP dose response curve in n=553 subjects, r=-0.249.

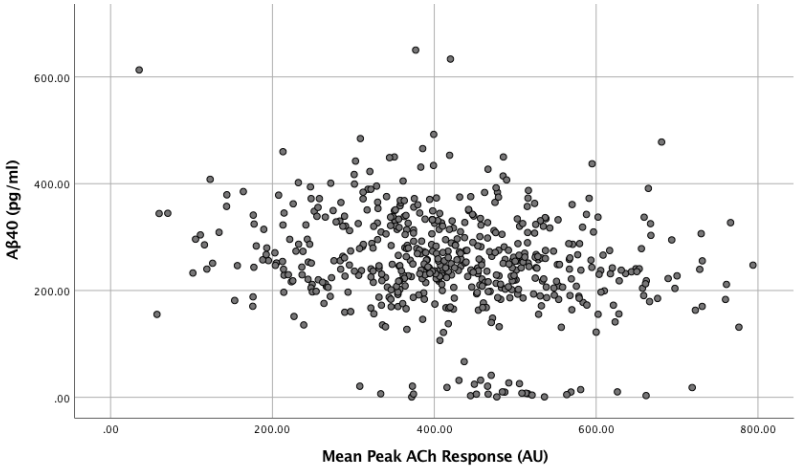

Figure 3-10: Scatter plot of plasma β-amyloid 40 with the average of last 3 readings on the ACh dose response curve in n=555 subjects, r=-0.211.

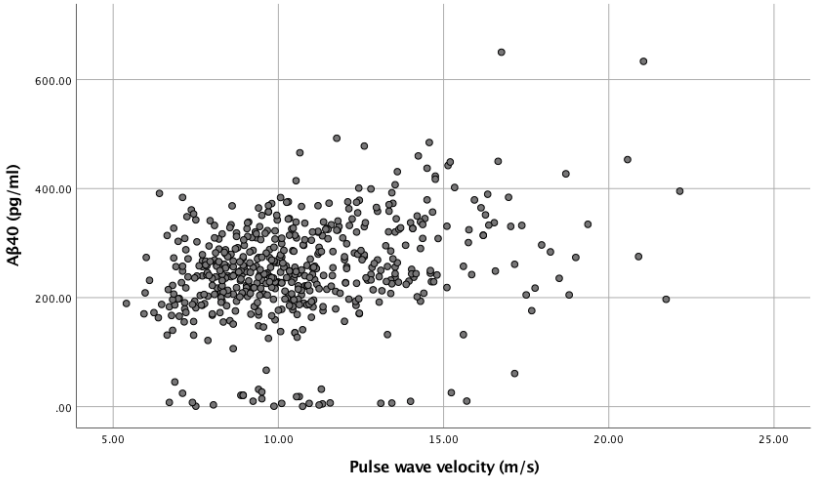

Figure 3-11: Scatter plot of plasma β-amyloid 40 with pulse wave velocity in n=540 subjects, r=0.282.

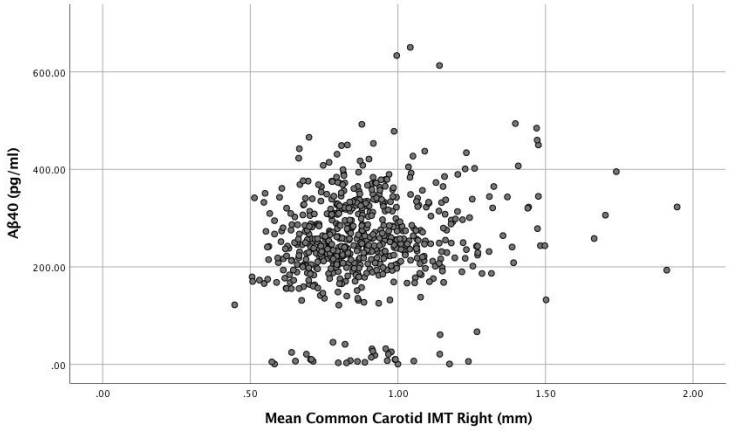

Figure 3-12: Scatter plot of plasma β-amyloid 40 with right sided common carotid IMT in n=619 subjects, r=0.14

Using univariate correlations, the analysis above looks at the association of β-amyloid 40 and 42 with markers of vascular functional and structural health. After adjusting for multiple comparisons using the Bonferroni method, only β-amyloid 40 showed significant correlations with vascular markers. A significant positive correlation was found when looking at β-amyloid 40 and pulse wave velocity as well as right-right sided CC intima media thickness. Significant negative correlations were found between β-amyloid 40 and the mean maximum response to SNP and Ach iontophoresis. While β-amyloid 42 correlated with some vascular markers at the standard level of significance, $p<0.05$, none of these associations reached the newly defined threshold for significance.

3.5.2.2 *Regression Model: SUMMIT Baseline Cohort*

The next step was to determine whether the associations described above persisted after adjusting for conventional CVD risk factors. This linear regression model adjusted for cardiovascular risk factors present in the Framingham and ASSIGN risk score calculators: age, gender, diabetes status, SBP, total cholesterol and HDL cholesterol. Additionally, in order to determine whether the effect of β-amyloid was independent of any factors identified by the analysis in chapter 2 such as renal function or insulin use, this model also adjusted for independent predictors of plasma β-amyloid 40 and 42. For β-amyloid 40 in the SUMMIT baseline cohort these were eGFR and diuretic use, and for β-amyloid 42 these were eGFR, diuretic use and LDL cholesterol. Beta refers to the standardised regression coefficient to allow for easy comparisons between models. Each row with corresponding β-amyloid column in the table below refer to an individual regression model.

Table 3-4: Summary of individual regression models with independent variables age, gender, diabetes status, SBP, total cholesterol, HDL cholesterol, β-amyloid 40 or 42 and either eGFR + diuretic use or eGFR + diuretic use + LDL cholesterol.

Dependent Variable	**Aβ40 (pg/ml)**		**Aβ42 (pg/ml)**	
	Beta	p-value	Beta	p-value
Reactive Hyperaemia Peak Perfusion (PU)	**0.116**	**0.019**	**-0.107**	**0.035**
Reactive Hyperaemia Index (EndoPAT units)	0.003	0.952	-0.021	0.618
Pulse Wave Velocity (m/s)	**0.17**	**5.00E-06**	0.023	0.559
Common Carotid IMT Right (mm)	0.033	0.413	0.005	0.906
Mean Bulb IMT Right (mm)	0.027	0.557	-0.01	0.828
Common Carotid IMT Left (mm)	-0.007	0.868	-0.047	0.271
Mean Bulb IMT Left (mm)	0.083	0.076	0.076	0.11
Peak SNP Response (PU)	**-0.175**	**9.10E-05**	0.033	0.473
Peak ACh Response (PU)	**-0.154**	**0.001**	0.011	0.813

After adjusting for conventional risk factors described above as well as clinical determinants of plasma β-amyloid 40 and 42, β-amyloid was 40 was found to be significantly associated with reactive hyperaemia, pulse wave velocity, peak SNP response and peak ACh response. β-amyloid 42 was only significantly associated with a reduced peak reactive hyperaemia perfusion response.

3.5.2.3 *Association of Aβ quartiles with clinically manifest CVD at enrolment into study*

Having investigated the association between plasma β-amyloid and biomarkers of cardiovascular health, the next step was to determine how plasma β-amyloid varies with different forms of clinically manifest CVD. The following cross tabulations refer to the association between SUMMIT baseline cohort β-amyloid quartiles, coronary heart disease, lower extremity arterial disease and cerebrovascular disease status at enrolment into the study. β-amyloid 40 quartiles are presented first. The Mantel Haenszel test for trend is a modified Chi-square test whereby significance suggests that association between the predictor and outcome variable is significantly different in the different levels of the conditional variable. To adjust for multiple analyses, the Bonferroni method was used to set the new threshold of significance at p<0.008.

Table 3-5: Cross tabulation summarising number of subjects with CHD in each β-amyloid 40 quartile at enrolment into study.

SUMMIT Baseline Cohort		Any CHD		Total
		No CHD	CHD	
Aβ40 Quartile (pg/ml)	0.6-207.9	98	64	162
	208-251.7	109	51	160
	251.8 – 314.4	104	58	162
	314.5-650.2	97	62	159
Total		408	235	643

Mantel-Haenszel linear by linear association: $x^2(1)=0.017$, p=0.896

Table 3-6: Cross tabulation summarising number of subjects with LEAD in each β-amyloid 40 quartile at enrolment into study.

SUMMIT Baseline Cohort		Any LEAD		Total
		No LEAD	LEAD	
Aβ40 Quartile (pg/ml)	0.6-207.9	150	12	162
	208-251.7	144	16	160
	251.8 – 314.4	143	19	162
	314.5-650.2	118	41	159
Total		555	88	643

Mantel-Haenszel linear by linear association: $x^2(1)=21.78$, p=3E-6

Table 3-7: Cross tabulation summarising number of subjects with cerebrovascular disease in each β-amyloid 40 quartile at enrolment into study.

SUMMIT Baseline Cohort		Cerebrovascular Disease		Total
		No	Yes	
Aβ40 Quartile (pg/ml)	0.6-207.9	150	12	162
	208-251.7	143	17	160
	251.8 – 314.4	149	13	162
	314.5-650.2	131	28	159
Total		573	70	643

Mantel-Haenszel linear by linear association: $x^2(1)=6.42$, p=0.011

Table 3-8: Cross tabulation summarising number of subjects with CHD in each β-amyloid 42 quartile at enrolment into study.

SUMMIT Baseline Cohort		Any CHD		Total
		No CHD	CHD	
Aβ42 Quartile (pg/ml)	0.06-9.64	97	64	161
	9.65-12.1	111	50	161
	12.2-14.78	112	49	161
	14.79-37.43	88	72	160
Total		408	235	643

Mantel-Haenszel linear by linear association: $x^2(1)=0.78$, p=0.378

Table 3-9: Cross tabulation summarising number of subjects with LEAD in each β-amyloid 42 quartile at enrolment into study.

SUMMIT baseline cohort		Any LEAD		Total
		No LEAD	LEAD	
Aβ42 Quartile (pg/ml)	0.06-9.64	138	23	161
	9.65-12.1	140	21	161
	12.2-14.78	142	19	161
	14.79-37.43	137	23	160
Total		557	86	643

Mantel-Haenszel linear by linear association: $x^2(1)=0.007$, p=0.934

Table 3-10: Cross tabulation summarising number of subjects with cerebrovascular disease in each β-amyloid 42 quartile at enrolment into study.

SUMMIT Baseline Cohort		Cerebrovascular Disease		Total
		No	Yes	
Aβ42 Quartile (pg/ml)	0.06-9.64	146	15	161
	9.65-12.1	148	13	161
	12.2-14.78	144	17	161
	14.79-37.43	135	25	160
Total		573	70	643

Mantel-Haenszel linear by linear association: $x^2(1)=3.78$, p=0.052

The above analysis used a variation of the Chi-Square test, the Mantel-Haenszel test for trend to determine whether a significant association exists between β-amyloid quartiles and different types of clinically manifest CVD at enrolment into the study. At the new threshold for significance (p<0.008) increasing β-amyloid 40 quartiles were significantly associated with increasing numbers of LEAD cases.

3.5.2.4 *Analysis of Plasma β-amyloid Levels and Cardiovascular Outcomes*

Having performed a cross-sectional type analysis looking at clinically manifest CVD at enrolment into the study in relation to plasma beta amyloid levels, the next step was to analyse any associations with clinically manifest CVD over a prospective follow up period. Ideally, to determine the association between β-amyloid and cardiovascular outcomes, a more robust analysis would have been to use Cox regression taking into account time to event. However, due to the lack of information about time of event within the available database, a logistic regression model was used instead of a survival analysis model. The following table summarises the results of a binary logistic regression model looking at patient outcomes accumulated over a period of 4-6 years of follow up. This model adjusted for risk factors used in Framingham and ASSIGN risk score calculators. These include age, gender, diabetes status, total cholesterol, HDL cholesterol and systolic blood pressure as well as β-amyloid 40 and 42.

Table 3-11: Summary of individual binary logistic regression models with independent variables age, gender, diabetes status, total cholesterol, HDL cholesterol, systolic BP and β-amyloid 40 or 42.

Outcome – Dependent Variable	**Aβ40 (pg/ml)**		**Aβ42 (pg/ml)**	
	Exp(B)	p-value	Exp(B)	p-value
Death from any cause (n=18)	1.00	0.879	0.966	0.515
Acute MI (n=14)	0.997	0.235	0.943	0.321
Unstable Angina (n=20)	0.999	0.630	0.962	0.445
Stroke (n=6)	1.001	0.855	1.094	0.258
TIA (n=12)	0.996	0.280	0.983	0.777
Intermittent Claudication (n=17)	1.002	0.574	0.981	0.748
Composite Cardiovascular Outcomes (n=70)	1.000	0.792	0.957	0.625

Using binary logistic regression with cardiovascular individual or composite cardiovascular outcomes as dependent variables and established CVD risk factors as well as β-amyloid 40 and 42 as independent variables, neither β-amyloid 40 nor β-amyloid 42 were found to be significantly independently associated with any cardiovascular outcome in the SUMMIT baseline cohort.

3.5.3 Analysis of SUMMIT Subjects Without T2DM

In order to determine whether some of the associations found in the SUMMIT baseline cohort were also present in subjects with and without diabetes individually, the initial analysis was repeated in subjects without diabetes and subjects with diabetes individually. Although diabetes status was not a significant independent predictor of β-amyloid levels in regression models in the previous chapter, based on findings from our previous animal study as well as studies from other research groups, diabetes status was deemed to be of clinical importance.

3.5.3.1 *SUMMIT Subjects Without Diabetes: Correlations of Plasma β-amyloid with Biomarkers of Cardiovascular Health*

The first step was to analyse plasma β-amyloid in relation to biomarkers of cardiovascular health in groups of subjects with and without diabetes. The following analysis will first display results for SUMMIT subjects without diabetes, followed by results for those with diabetes. Depending on variable distribution, either Pearson or Spearman correlation was used to determine whether a significant univariate correlation exists between plasma β-amyloid 40 or 42 and biomarkers of cardiovascular health. Scatterplots for significant associations are displayed below. The Bonferroni method was used to correct for multiple comparisons by dividing the standard p value 0.05 by the number of comparisons made, with the new threshold for significance set at $p<0.003$.

Table 3-12: Univariate correlations of plasma β-amyloid with markers of vascular structural or functional change in SUMMIT subjects without diabetes.

	Correlation	**Aβ40 (pg/ml)**	**Aβ42 (pg/ml)**
Reactive Hyperaemia Index (EndoPAT)	Correlation Coefficient	0.084	0.001
	Sig.	0.201	0.992
	N	235	235
Reactive Hyperaemia Peak Perfusion (PU)	Correlation Coefficient	0.039	-0.091
	Sig.	0.584	0.206
	N	195	196
Pulse wave velocity (m/s)	Correlation Coefficient	0.268	0.037
	Sig.	**4.60E-05**	0.585
	N	225	226
Mean CC IMT Right (mm)	Correlation Coefficient	0.216	0.106
	Sig.	**0.001**	0.104
	N	238	238
Mean CC Bulb IMT Right (mm)	Correlation Coefficient	0.046	-0.119
	Sig.	0.523	0.093
	N	199	199
Mean CC IMT Left (mm)	Correlation Coefficient	0.109	-0.003
	Sig.	0.093	0.958
	N	237	237
Mean CC Bulb IMT Left (mm)	Correlation Coefficient	0.107	-0.046
	Sig.	0.123	0.51
	N	208	208
Mean Peak ACh Response (PU)	Correlation Coefficient	-0.054	0.079
	Sig.	0.434	0.246
	N	214	215
Mean Peak SNP Response (PU)	Correlation Coefficient	-0.099	0.15
	Sig.	0.153	0.029
	N	212	213

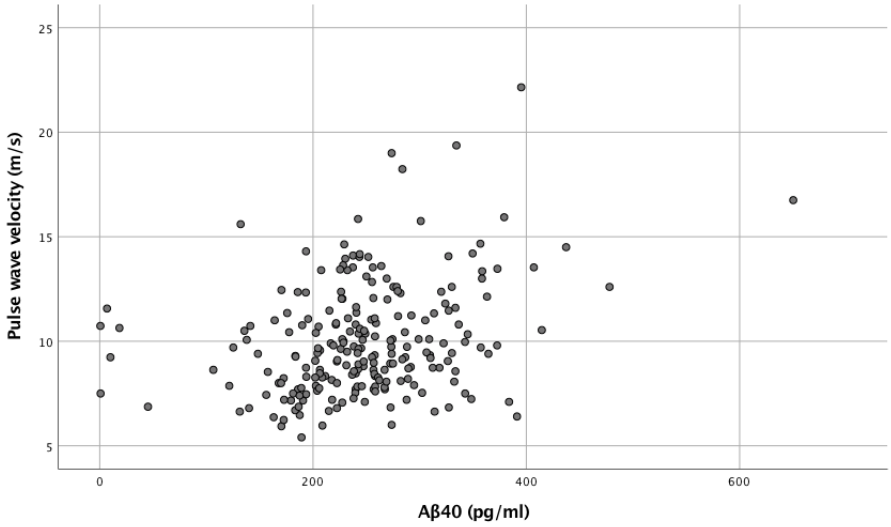

Figure 3-13: Scatter plot of β-amyloid 40 with pulse wave velocity in SUMMIT subjects without diabetes, n=225, r=0.268.

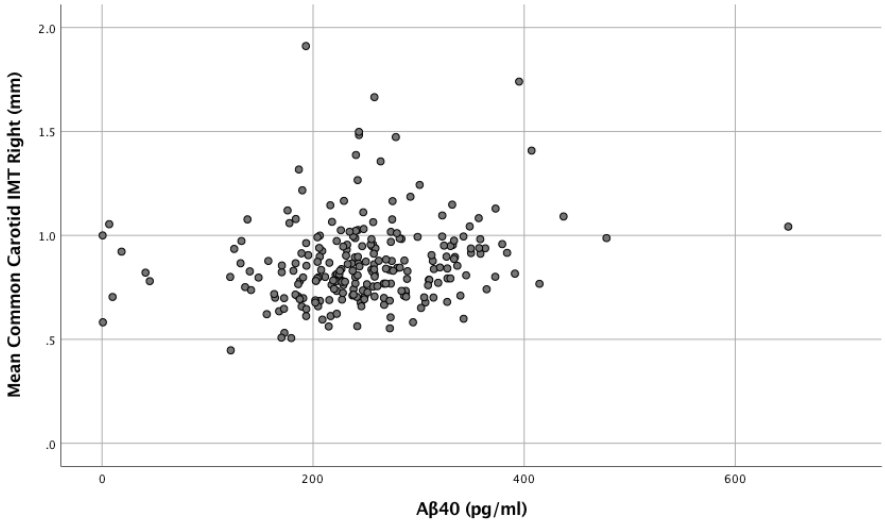

Figure 3-14: Scatter plot of β-amyloid 40 with right-sided mean common carotid IMT in SUMMIT subjects without diabetes, n=238, r=0.216.

A univariate analysis of plasma β-amyloid with markers of vascular structural and functional health in subjects without T2DM revealed 2 significant positive correlations. β-amyloid 40 was found to correlate positively with pulse wave velocity as well as mean right-sided CC IMT. No significant correlations were found when looking at β-amyloid 42 after Bonferroni adjustment of the p-value.

3.5.3.2 *Regression Model: SUMMIT Subjects without Diabetes*

The next step was to determine whether any significant associations discovered in univariate correlation of plasma β-amyloid with biomarkers of vascular health persisted after adjusting for common CVD risk factors. These models included recognised CVD risk factors included in the Framingham and ASSIGN risk score calculators and adjusted for age, gender, systolic blood pressure, total cholesterol and HDL cholesterol. Where the effect of β-amyloid 40 was investigated, the model also adjusted for eGFR and height (age already included), as these were significant predictors of β-amyloid in Chapter 2. Where the model investigated the effect of β-amyloid 42, eGFR was also included in the model. Each row with corresponding β-amyloid column in the table below represents an individual regression model.

Table 3-3-13: Summary of individual linear regression models with independent variables age, gender, SBP, total cholesterol, HDL cholesterol as well as β-amyloid 40 or 42 and eGFR, Height or eGFR respectively.

Vascular Measurement – Dependent Variable	**Aβ40 (pg/ml)**		**Aβ42 (pg/ml)**	
	Beta	p-value	Beta	p-value
Reactive Hyperaemia Peak Perfusion (PU)	0.047	0.54	-0.14	0.066
Reactive Hyperaemia Index (EndoPAT units)	0.068	0.336	-0.052	0.457
Pulse Wave Velocity (m/s)	**0.17**	**0.003**	0.011	0.84
Common Carotid IMT Right (mm)	0.062	0.324	0.079	0.195
Mean Bulb IMT Right (mm)	-0.052	0.464	-0.113	0.112
Common Carotid IMT Left (mm)	-0.021	0.75	-0.022	0.733
Mean Bulb IMT Left (mm)	0.083	0.249	-0.019	0.766
Mean SNP Response (PU)	-0.031	0.675	**0.123**	**0.016**
Mean ACh Response (PU)	-0.005	0.945	0.173	0.091

The above analysis shows that after adjusting for common CVD risk factors as well as clinical determinants of β-amyloid identified in chapter 2, higher β-amyloid 40 levels were significantly independently associated with pulse wave velocity. Interestingly, the univariate correlation between β-amyloid 42 and SNP was not significant at the Bonferroni adjusted p-value threshold, however, in the above linear regression model β-amyloid 42 was found to be significantly and independently associated with the mean peak response to SNP iontophoresis.

3.5.3.3 *Analysis of Plasma β-amyloid in relation to Clinically Manifest Cardiovascular Disease – SUMMIT Subjects Without Diabetes*

Having investigated the relationship between plasma β-amyloid and biomarkers of cardiovascular health or disease risk, the next step was to determine whether any association exists between plasma β-amyloid levels and clinically manifest CVD. The following cross tabulations summarise the associations between plasma β-amyloid 40 and 42 quartiles and different forms of CVD at enrolment into study. The Mantel Haenszel test for trend is a modified Chi-square test whereby significance suggests that association between the predictor and outcome variable is significantly different in the different levels of the conditional variable. Using the Bonferroni method, the new threshold for significance was again set at p<0.008.

Table 3-14: Cross tabulation summarising number of cases of CHD in each β-amyloid 40 quartile at enrolment into the study in SUMMIT subjects without diabetes.

SUMMIT Subjects Without T2DM		**Any CHD**		**Total**
		No	Yes	
Aβ40 Quartile (pg/ml)	0.60-205.34	36	25	61
	205.35-243.4	41	20	61
	243.5-287.3	33	28	61
	287.4-650.2	42	19	61
Total		152	92	244

Mantel-Haenszel linear by linear association: $x^2(1)=0.348$, p=0.556

Table 3-15: Cross tabulation summarising number of cases of LEAD in each β-amyloid 40 quartile at enrolment into the study in SUMMIT subjects without diabetes

SUMMIT Subjects Without T2DM		**Any LEAD**		**Total**
		No	Yes	
Aβ40 Quartile (pg/ml)	0.60-205.34	59	2	61
	205.35-243.4	56	5	61
	243.5-287.3	53	8	61
	287.4-650.2	53	8	61
Total		221	23	244

Mantel-Haenszel linear by linear association: $x^2(1)=4.217$, p=0.04

Table 3-16: Cross tabulation summarising number of cases of cerebrovascular disease in each β-amyloid 40 quartile at enrolment into the study in SUMMIT subjects without diabetes.

SUMMIT Subjects Without T2DM		Cerebrovascular Disease		Total
		No	Yes	
Aβ40 Quartile (pg/ml)	0.60-205.34	57	4	61
	205.35-243.4	55	6	61
	243.5-287.3	53	8	61
	287.4-650.2	48	13	61
Total		213	31	244

Mantel-Haenszel linear by linear association: $x^2(1)=6.19$, p=0.013

Table 3-17: Cross tabulation summarising number of cases of CHD in each β-amyloid 42 quartile at enrolment into the study in subjects without diabetes.

SUMMIT Subjects Without Diabetes		Any CHD		Total
		No	Yes	
Aβ42 Quartile (pg/ml)	0.21-9.15	36	26	62
	9.16-11.57	42	18	60
	11.58-14.3	39	22	61
	14.4-27.98	35	26	61
Total		152	92	244

Mantel-Haenszel linear by linear association: $x^2(1)=0.078$, p=0.780

Table 3-18: Cross tabulation summarising number of cases of LEAD in each β-amyloid 42 quartile at enrolment into the study, in SUMMIT subjects without diabetes.

SUMMIT Subjects Without T2DM		Any LEAD		Total
		No	Yes	
Aβ42 Quartile (pg/ml)	0.21-9.15	54	8	62
	9.16-11.57	58	2	60
	11.58-14.3	54	7	61
	14.4-27.98	56	5	61
Total		222	22	244

Mantel-Haenszel linear by linear association: $x^2(1)=0.144$, p=0.704

Table 3-19: Cross tabulation summarising number of cases of cerebrovascular disease in each β-amyloid 42 quartile at enrolment into the study in SUMMIT subjects without diabetes.

SUMMIT Subjects Without T2DM		**Cerebrovascular Disease**		**Total**
		No	Yes	
Aβ42 Quartile (pg/ml)	0.21-9.15	55	7	62
	9.16-11.57	58	2	60
	11.58-14.3	51	10	61
	14.4-27.98	50	11	61
Total		214	30	244

Mantel-Haenszel linear by linear association: $x^2(1)=3.08$, p=0.079

Using the Mantel-Haenszel test for trend, no significant associations were found between β-amyloid quartiles and CHD, LEAD or cerebrovascular disease.

3.5.4 Analysis of SUMMIT Subjects With T2DM

3.5.4.1 *Correlation of Plasma β-amyloid with biomarkers of cardiovascular health in the SUMMIT diabetic cohort*

The same analysis performed above was then repeated for SUMMIT subjects with T2DM. In order to determine whether plasma β-amyloid was associated with any biomarkers of cardiovascular health/disease risk, univariate correlations were examined. Depending on the distribution of variables, either Spearman or Pearson correlations were used. As before, the Bonferroni method was used to correct for multiple comparisons, with a new threshold for significance set at $p<0.003$. Scatterplots for significant associations are displayed below.

Table 3-20: Summary of univariate correlations of β-amyloid 40 and 42 with markers of structural or functional vascular changes in SUMMIT subjects with diabetes

Vascular Biomarker	Correlation	Aβ40 (pg/ml	Aβ42 (pg/ml)
Reactive Hyperaemia Index (EndoPAT units)	Correlation Coefficient	-0.068	-0.052
	Sig.	0.193	0.321
	N	370	370
Reactive Hyperaemia Peak Perfusion (PU)	Correlation Coefficient	0.128	-0.159
	Sig.	0.031	0.007
	N	285	285
Pulse wave velocity (m/s)	Correlation Coefficient	0.289	0.145
	Sig.	**1.74E-07**	0.01
	N	315	315
Mean Common Carotid IMT Right (mm)	Correlation Coefficient	0.088	0.043
	Sig.	0.085	0.403
	N	381	380
Mean CC Bulb IMT Right (mm)	Correlation Coefficient	0.021	-0.006
	Sig.	0.72	0.915
	N	300	300
Mean Common Carotid IMT Left (mm)	Correlation Coefficient	0.092	-0.02
	Sig.	0.071	0.695
	N	384	384
Mean CC Bulb IMT Left (mm)	Correlation Coefficient	0.105	0.056
	Sig.	0.063	0.321

	N	315	316
Mean Peak ACh Response (PU)	Correlation Coefficient	-0.282	-0.09
	Sig.	**1.17E-07**	0.096
	N	341	340
Mean Peak SNP Response (PU)	Correlation Coefficient	-0.311	-0.054
	Sig.	**4.46E-09**	0.321
	N	341	340

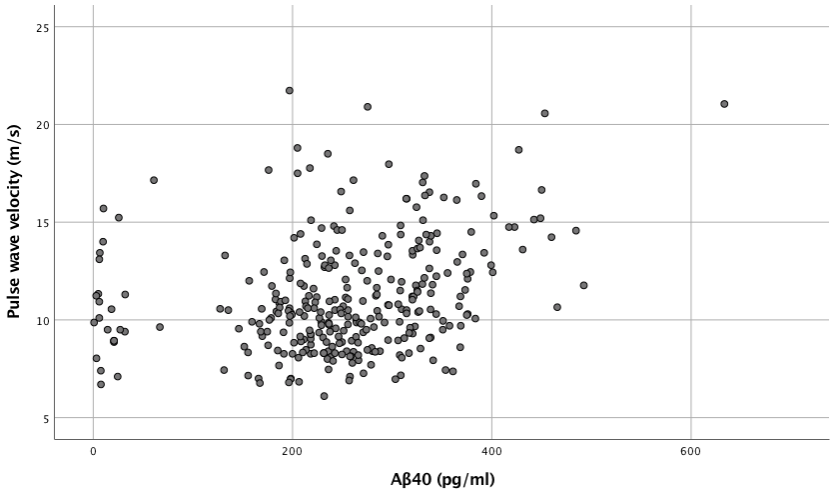

Figure 3-15: Scatter plot of plasma β-amyloid 40 with pulse wave velocity in SUMMIT subjects with diabetes, n=315, r=0.289.

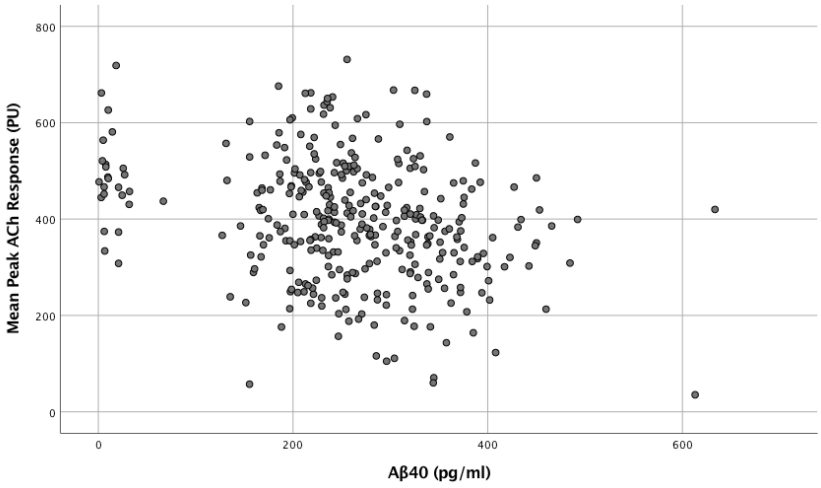

Figure 3-16: Scatter plot of plasma β-amyloid 40 with peak ACh response in SUMMIT subjects with diabetes, n=341, r=-0.282.

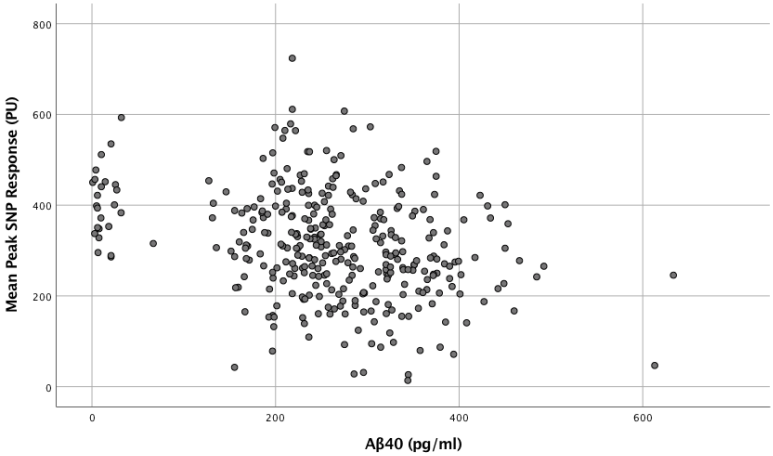

Figure 3-17: Scatter plot of plasma β-amyloid 40 with peak SNP response in SUMMIT subjects with diabetes, n=341, r=-0.311.

The above univariate analysis in subjects with T2DM shows that significant negative correlations exist between β-amyloid 40 and peak response to ACh as well as SNP iontophoresis. Additionally, as seen in previous univariate analyses, a significant positive correlation is seen with β-amyloid 40 and pulse wave velocity.

3.5.4.2 *Regression: SUMMIT Subjects With T2DM*

To determine whether the associations of plasma β-amyloid with biomarkers of cardiovascular disease persist even after adjusting for common CVD risk factors, a linear regression model was used. These models adjust for age, gender, systolic blood pressure, total cholesterol, HDL cholesterol and smoking status. Where β-amyloid 40 was included in the model, additional factors adjusted for included eGFR, insulin use and diuretic use. Where β-amyloid 42 was included in the model, factors adjusted for also included eGFR and insulin use. Each row with corresponding β-amyloid column in the table below represents an individual regression model.

Table 3-21: Summary of linear regression models with independent variables age, gender, systolic blood pressure, total cholesterol, HDL cholesterol an either β-amyloid 40 or 42, with eGFR, insulin use and diuretic use or eGFR and insulin use respectively.

Vascular Measurement – Dependent Variable	**Aβ40 (pg/ml)**		**Aβ42 (pg/ml)**	
	Beta	p-value	Beta	p-value
Reactive Hyperaemia Peak Perfusion (PU)	**0.179**	**0.008**	-0.105	0.113
Reactive Hyperaemia Index (EndoPAT units)	-0.027	0.636	-0.007	0.903
Pulse Wave Velocity (m/s)	**0.153**	**0.003**	0.03	0.559
Common Carotid IMT Right (mm)	0.017	0.76	-0.05	0.355
Mean Bulb IMT Right (mm)	0.055	0.382	0.032	0.604
Common Carotid IMT Left (mm)	-0.001	0.992	-0.059	0.102
Mean Bulb IMT Left (mm)	0.082	0.19	0.101	0.1
Mean Peak SNP Response (PU)	**-0.229**	**1.19E-04**	-0.034	0.568
Mean Peak ACh Response (PU)	**-0.263**	**1.10E-05**	-0.04	0.499

In subjects with T2DM, a linear regression analysis showed that β-amyloid 40 was significantly and independently associated with peak reactive hyperaemia response, pulse wave velocity, peak response to SNP as well as ACh iontophoresis, even after adjusting for conventional CVD risk factors and clinical determinants of β-amyloid. β-amyloid 42 was not found to be independently associated with any of the markers of vascular functional or structural health.

3.5.4.3 *Analysis of Plasma β-amyloid in relation to Clinically Manifest Cardiovascular Disease – SUMMIT Subjects With T2DM*

The following analysis was used to investigate the association between plasma β-amyloid and clinically manifest CVD. Due to small numbers of accumulated outcomes, no follow up outcome analysis was performed. The Mantel Haenszel test for trend is a modified Chi-square test whereby significance suggests that association between the predictor and outcome variable is significantly different in the different levels of the conditional variable. As previous, the Bonferroni method was used to set the new threshold for significance at p<0.008.

Table 3-22: Cross tabulation summarising number of cases of CHD in each β-amyloid 40 quartile, in SUMMIT subjects with T2DM at enrolment into study.

SUMMIT Subjects With T2DM		**Any CHD**		**Total**
		No	Yes	
Aβ40 Quartile (pg/ml)	0.80-211.3	62	38	100
	211.4-258.7	73	27	100
	258.8-325.9	65	35	100
	326-633.4	56	43	99
Total		256	143	399

Mantel-Haenszel linear by linear association: $x^2(1)=1.263$, p=0.261

Table 3-23: Cross tabulation summarising number of cases of LEAD in each β-amyloid 40 quartile at enrolment into the study in SUMMIT subjects with T2DM.

SUMMIT Subjects With T2DM		**Any LEAD**		**Total**
		No	Yes	
Aβ40 Quartile (pg/ml)	0.80-211.3	91	9	100
	211.4-258.7	89	11	100
	258.8-325.9	87	13	100
	326-633.4	67	32	99
Total		334	65	399

Mantel-Haenszel linear by linear association: $x^2(1)=18.776$, p=1.5E-5

Table 3-24: Cross tabulation summarising number of cases of cerebrovascular disease in each β-amyloid 40 quartile at enrolment into the study, in SUMMIT subjects with T2DM.

SUMMIT Subjects With T2DM		Cerebrovascular Disease		Total
		No	Yes	
Aβ40 Quartile (pg/ml)	0.80-211.3	92	8	100
	211.4-258.7	90	10	100
	258.8-325.9	94	6	100
	326-633.4	84	15	99
Total		360	39	399

Mantel-Haenszel linear by linear association: $x^2(1)=1.699$, p=0.192

Table 3-25: Cross tabulation summarising number of cases of CHD in each β-amyloid 42 quartile at enrolment into study in SUMMIT subjects with T2DM.

SUMMIT Subjects With T2DM		Any CHD		Total
		No	Yes	
Aβ42 Quartile (pg/ml)	0.06-9.8	61	39	100
	9.9-12.5	70	30	100
	12.6-14.9	72	28	100
	15-37.43	53	46	99
Total		256	143	399

Mantel-Haenszel linear by linear association: $x^2(1)=0.878$, p=0.349

Table 3-26: Cross tabulation summarising number of cases of LEAD in each β-amyloid 42 quartile at enrolment into study, in SUMMIT subjects with T2DM.

SUMMIT Subjects With T2DM		Any LEAD		Total
		No	Yes	
Aβ42 Quartile (pg/ml)	0.06-9.8	85	15	100
	9.9-12.5	82	18	100
	12.6-14.9	88	12	100
	15-37.43	80	19	99
Total		335	64	399

Mantel-Haenszel linear by linear association: $x^2(1)=0.156$, p=0.693

Table 3-27: Cross tabulation summarising number of cases of cerebrovascular disease in each β-amyloid 42 quartile at enrolment into study, in SUMMIT subjects with T2DM.

SUMMIT Subjects With T2DM		Cerebrovascular Disease		Total
		No	Yes	
Aβ42 Quartile (pg/ml)	0.06-9.8	90	10	100
	9.9-12.5	93	7	100
	12.6-14.9	91	9	100
	15-37.43	85	14	99
Total		359	40	399

Mantel-Haenszel linear by linear association: $x^2(1)=1.136$, p=0.282

In the above analysis, a modified version of the Chi-Square test was used to investigate the relationship between β-amyloid quartiles and different types of clinically manifest CVD at enrolment into the study. A significant association was seen between increasing β-amyloid 40 quartiles and number of LEAD cases, where the number of cases is seen to increase steadily with increasing β-amyloid quartiles.

3.5.5 Summary of key findings from the SUMMIT cohort:

3.5.5.1 *SUMMIT Baseline Cohort*

Linear regression analysis showed that higher β-amyloid 40 levels were significantly and independently associated with increased peak reactive hyperaemia response to temporary blood flow occlusion and increased pulse wave velocity. Increasing β-amyloid levels 40 were also significantly and independently associated with reduced peak perfusion response to SNP and ACh iontophoresis. β-amyloid 42 was significantly and independently associated with reduced reactive hyperaemia response following temporary blood flow occlusion.

When looking at clinically manifest CVD at time of enrolment into the study, increasing β-amyloid 40 levels appear to be associated with increased numbers of LEAD cases. No associations are seen with β-amyloid 42. No significant association were seen with β-amyloid and adverse cardiovascular outcomes or overall mortality.

3.5.5.2 *SUMMIT Non-T2DM Cohort*

Linear regression analysis revealed that higher β-amyloid 40 levels were significantly and independently associated with increased pulse wave velocity and higher β-amyloid 42 levels were significantly associated with increased peak response to SNP iontophoresis.

3.5.5.3 *SUMMIT T2DM Cohort*

Linear regression analysis revealed significant associations between β-amyloid 40 and increased reactive hyperaemia response to temporary blood flow occlusion, pulse wave velocity and reduced peak response to SNP and ACh iontophoresis. Additionally, increasing β-amyloid 40 levels were also associated with increasing numbers of LEAD cases at enrolment into study. No significant associations were reported for β-amyloid 42.

3.5.6 Sub-Group Analysis By Centre of Recruitment

Having completed the analysis of the baseline SUMMIT cohort as well as subgroup analysis looking at subjects with and without diabetes, the next step was to perform a subgroup analysis looking at individual centres of recruitment separately. This was done, as subjects recruited in different centres differed significantly in terms of baseline characteristics as well as β-amyloid levels. The following table summarises baseline patient data for each centre of recruitment. Where a significant difference was found between centres of recruitment, the level of statistical significance is illustrated. Mean comparisons were performed using either the Mann-Whitney or Independent T-tests depending on distribution of variables. * = p<0.05, ** = p<0.01, *** = p<0.001.

*Table 3-28: Summary of baseline patient characteristics in each centre of recruitment, significant differences are illustrated with *.*

Baseline Characteristics	Dundee Baseline Cohort	Exeter Baseline Cohort
N	376	276
Male sex n (%)	220 (59%)	189 (68%)
Age (SD)	65.2 (8.4)	66.9 (8.9)**
BMI (kg/m2)	30.7 (5.5)	29 (5.1)***
Medication		
Statin use	273 (73%)	185 (67%)
Antihypertensive use	255 (68%)	163 (59%)*
Blood Pressure		
SBP	133.6 (17.2)	130 (15.6)*
DBP	76.8 (8.7)	73.6 (8.5)***
Metabolic parameters		
HbA1c mmol/mol	51.7 (15.0)	51.5 (14.8)
Total Cholesterol mmol/l	4.1 (1.0)	4.4 (1.1) ***
LDL Cholesterol mmol/l	2.1 (0.9)	2.4 (1.0) ***
HDL Cholesterol mmol/l	1.3 (0.4)	1.4 (0.4) ***
Triglycerides mmol/l	1.7 (0.8)	1.4 (0.7) ***
Renal Function		
Serum Creatinine umol/l	81.2 (26.1)	84.9 (13.5)***
eGFR mL/min/1.73 m²	84.9 (23.2)	80.4 (17.1)**

3.5.6.1 *Comparison of the effect of cardiovascular disease and diabetes status on β-amyloid levels based on centre of recruitment*

To determine whether plasma β-amyloid levels differ in subjects with or without diabetes and with or without CVD across the different centres, the Mann-Whitney or Independent T test was used to make both within centre and between centre comparisons, depending on distribution of variables.

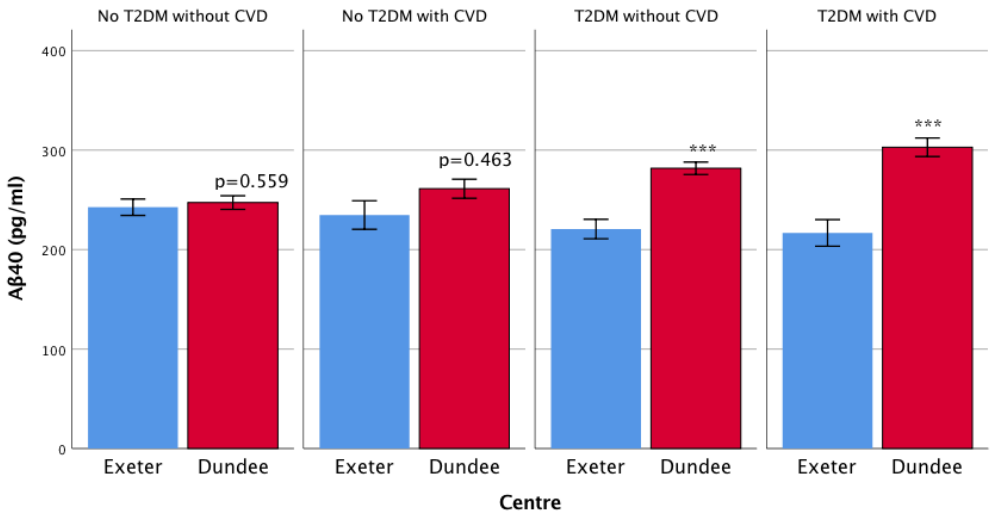

Figure 3-19: Bar graphs comparing β-amyloid 40 levels in different patient groups between centres of recruitment.

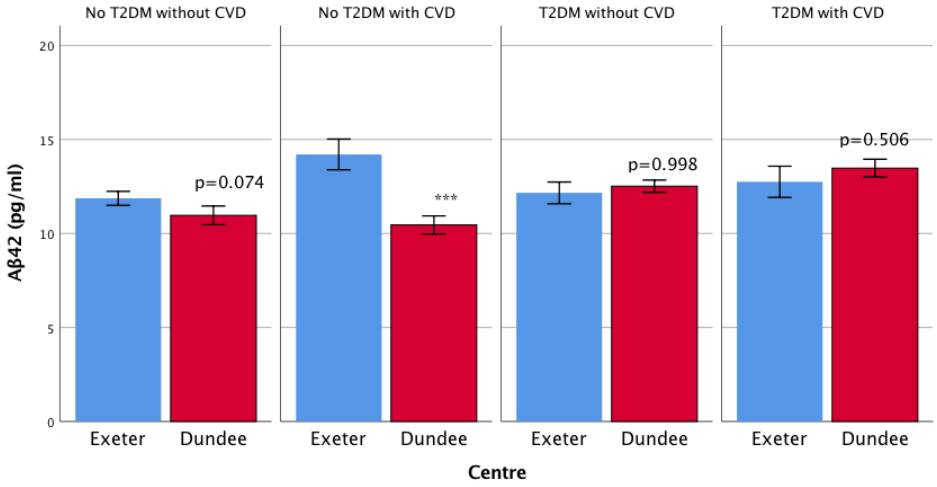

Figure 3-18: Bar graphs comparing β-amyloid 42 levels in different patient groups between centres of recruitment.

The above graphs show that significant differences in β-amyloid 40 levels exist between the Dundee and Exeter centres when looking at subjects with T2DM and with or without CVD. Additionally, a significant difference is also seen in β-amyloid 42 levels between Dundee and Exeter centres when looking at subjects without T2DM but with CVD. Together with significant differences in a number of baseline characteristics, this suggested that it would be of value to repeat some of the analyses done for the SUMMIT database separately for centres of recruitment.

3.5.7 Analysis of the Dundee Baseline Cohort

3.5.7.1 *Dundee Baseline Cohort: Correlations of Plasma β-amyloid With Vascular Biomarkers*

Results from the Dundee cohort are presented first. Univariate correlations were used to determine the association between plasma β-amyloid and markers of vascular structural or functional changes. Depending on the distribution of variables, either Spearman or Pearson correlations were used. As previously, the Bonferroni method was used to correct for multiple comparisons, by dividing 0.05 by the number of comparisons made (Bonferroni adjusted level of significance $p<0.003$). Scatterplots for significant univariate correlations are displayed below.

Table 3-29: Summary of univariate correlations between plasma β-amyloid and markers of vascular structural or functional change in the Dundee baseline cohort.

Vascular Measurement	Correlation	Aβ40	Aβ42
Reactive Hyperaemia Index (EndoPAT)	Correlation Coefficient	-0.069	-0.178
	Sig.	0.198	**0.001**
	N	347	347
Reactive Hyperaemia Peak Perfusion (PU)	Correlation Coefficient	-0.121	-0.282
	Sig.	0.066	**1.2E-5**
	N	232	233
Pulse wave velocity (m/s)	Correlation Coefficient	0.333	0.214
	Sig.	**7.67E-09**	**2.53E-4**
	N	287	288
Mean common carotid artery IMT Right (mm)	Correlation Coefficient	0.192	0.129
	Sig.	**3.24 E-4**	0.017
	N	347	346

Mean bulb IMT Right (mm)	Correlation Coefficient	0.2	0.017
	Sig.	**0.001**	0.784
	N	250	250
Mean common carotid artery IMT Left (mm)	Correlation Coefficient	0.123	0.008
	Sig.	0.021	0.875
	N	352	352
Mean bulb IMT Left (mm)	Correlation Coefficient	0.127	0.031
	Sig.	0.032	0.6
	N	283	284
Mean Peak SNP Response (mm)	Correlation Coefficient	-0.185	-0.088
	Sig.	**0.001**	0.133
	N	294	294
Mean Peak ACh Response	Correlation Coefficient	-0.143	-0.105
	Sig. (2-tailed)	0.014	0.07
	N	296	296

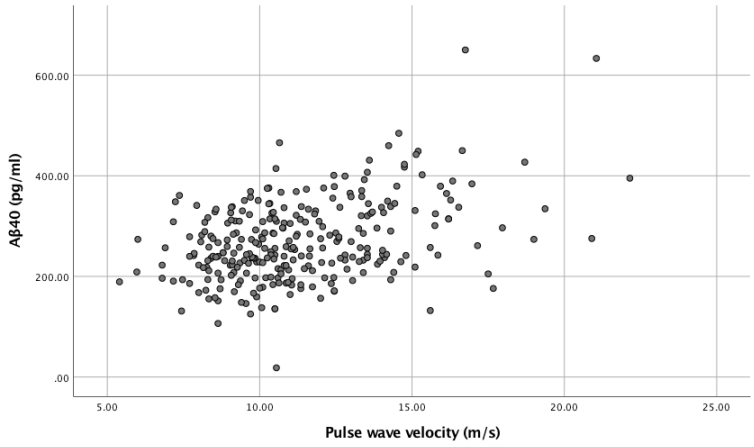

Figure 3-20: Scatter plot of β-amyloid 40 with pulse wave velocity in the Dundee baseline cohort, n=287, r=0.333.

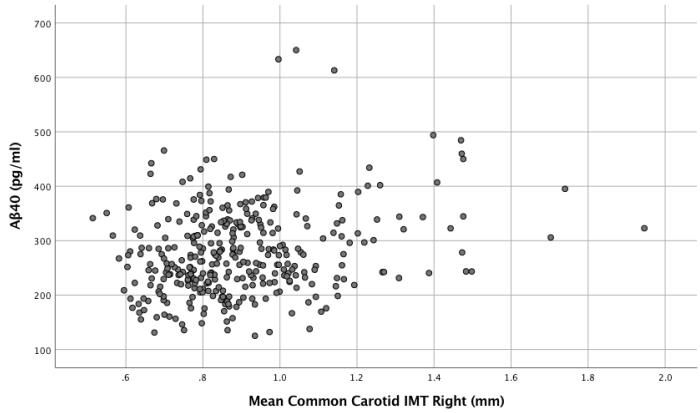

Figure 3-21: Scatter plot of β-amyloid 40 with right sided CC IMT in the Dundee baseline cohort, n=347, r=0.192

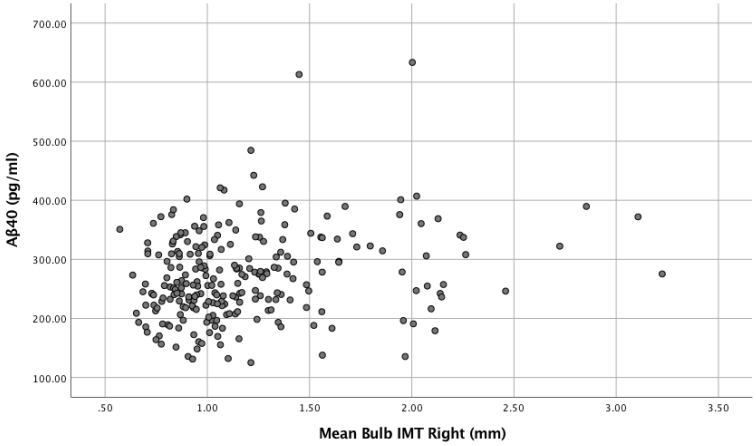

Figure 3-22: Scatter plot of β-amyloid 40 with right-sided mean common carotid bulb IMT in the Dundee baseline cohort, n=250, r=0.200.

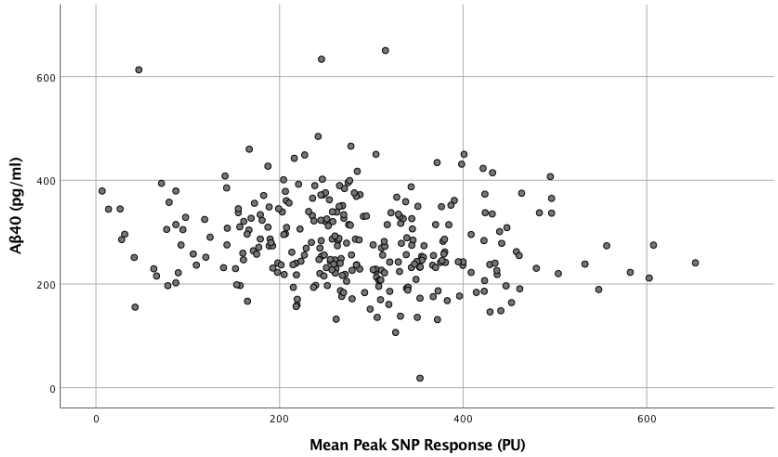

Figure 3-23: Scatter plot of β-amyloid 40 with peak response to SNP in the Dundee baseline cohort, n=294, r=-0.185

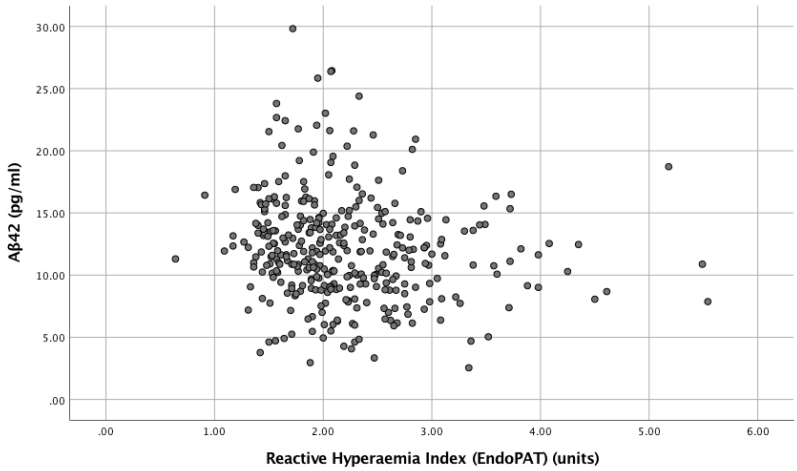

Figure 3-24: Scatter plot of β-amyloid 42 with reactive hyperaemia index as measured by EndoPAT in the Dundee baseline cohort, n=347, r=-0.178.

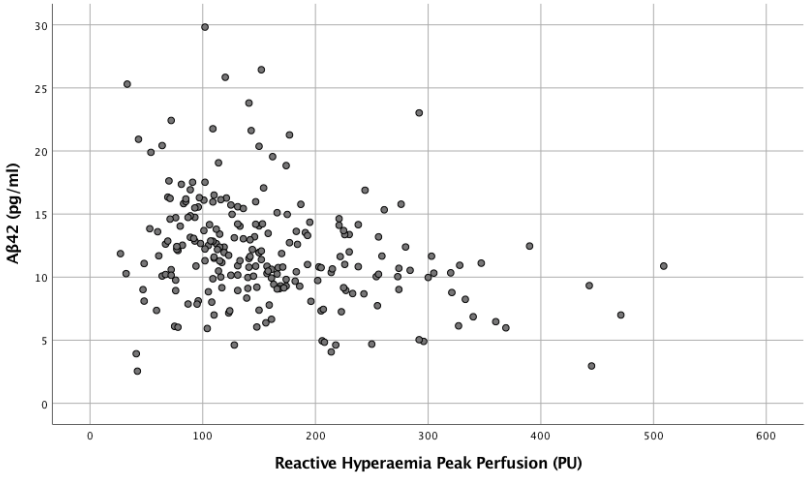

Figure 3-25: Scatter plot of β-amyloid 42 with reactive hyperaemia in the Dundee baseline cohort, n=233, r=-0.282

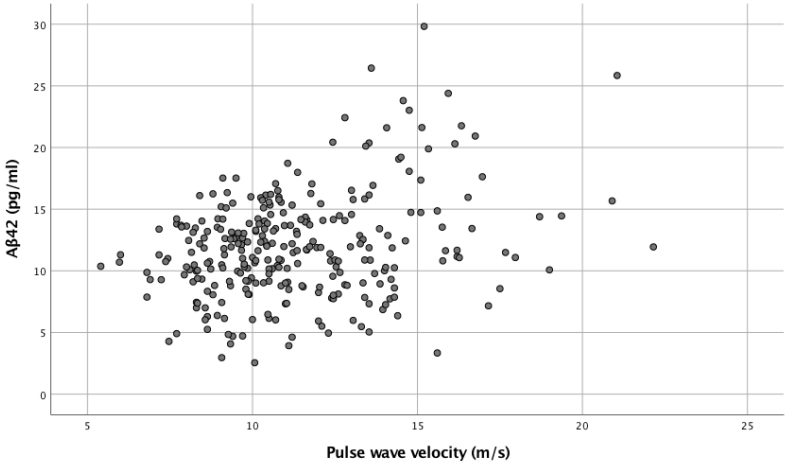

Figure 3-26: Scatter plot of β-amyloid 42 with pulse wave velocity in the Dundee baseline cohort, n=288, r=0.214

The above univariate correlations look at the Dundee subgroup separately. After Bonferroni adjustment to the new accepted threshold of significance at 0.003, significant positive correlations were found between β-amyloid 40 with pulse wave velocity, mean right-sided CC IMT and mean right sided bulb IMT, while a significant negative correlation was seen with the mean peak SNP response.

For β-amyloid 42, a significant negative correlation is seen with peak reactive hyperaemia response to temporary occlusion as well as RHI as measured by EndoPAT. Significant positive correlations can be seen between β-amyloid 42 with pulse wave velocity and right-sided CC IMT.

3.5.7.2 *Linear Regression: Dundee Baseline cohort*

As performed previously, a regression model was then built to determine whether any associations seen during univariate analysis persisted after adjusting for common CVD risk factors. This model adjusted for age, gender, diabetes status, total cholesterol, HDL cholesterol as well as factors identified as significant predictors of plasma β-amyloid in chapter 1. For β-amyloid 40 in the baseline cohort these were eGFR and diuretic use, and for β-amyloid 42 these were eGFR, diuretic use and LDL cholesterol. Beta refers to the standardised correlation coefficient. Each row and corresponding β-amyloid column represent a separate linear regression model with the dependent variable highlighted.

Table 3-30: Linear regression models with independent variables age, gender, diabetes status, total cholesterol, HDL cholesterol and either β-amyloid 40 or 42 with eGFR + diuretic use or eGFR + diuretic use + LDL cholesterol respectively.

Vascular Measurement – Dependent Variable	**Aβ40 (pg/ml)**		**Aβ42 (pg/ml)**	
	Beta	p-value	Beta	p-value
Reactive Hyperaemia Peak Perfusion (PU)	-0.033	0.693	**-0.215**	**0.009**
Reactive Hyperaemia Index (EndoPAT units)	0.015	0.801	-0.074	0.223
Pulse Wave Velocity (m/s)	**0.193**	**0.001**	0.116	0.048
Common Carotid IMT Right (mm)	0.082	0.185	-0.001	0.988
Mean Bulb IMT Right (mm)	0.119	0.096	-0.03	0.678
Common Carotid IMT Left (mm)	0.051	0.424	-0.058	0.358
Mean Bulb IMT Left (mm)	0.102	0.15	0.038	0.594
Mean SNP Response (PU)	-0.016	0.815	0.026	0.707
Mean ACh Response (PU)	-0.041	0.553	0.015	0.831

The above regression table summarises individual regression models. β-amyloid 40 was only found to be a significantly independently associated with pulse wave velocity, while β-amyloid 42 was seen to be significantly and independently associated with peak reactive hyperaemia response to temporary blood flow occlusion.

3.5.8 Analysis of Dundee Subjects Without T2DM

3.5.8.1 *Univariate Correlations in Dundee Subjects without T2DM*

The same analysis was then repeated in Dundee subjects without T2DM. The first step was to analyse the association between plasma β-amyloid and biomarkers of cardiovascular health. Depending on the distribution of variables, either the Mann-Whitney or independent T-tests were used. The Bonferroni method was used to correct for multiple comparisons, by dividing the p value 0.05 by number of comparisons made (adjusted p-value , p=0.003). Scatter plots for significant correlations are displayed below.

Table 3-31: Summary of univariate correlations of plasma β-amyloid with markers of vascular structural or functional changes in Dundee subjects without T2DM.

Vascular Biomarker	Correlation	Aβ40 (pg/ml)	Aβ42 (pg/ml)
Reactive Hyperaemia Index (EndoPAT units)	Correlation Coefficient	0.078	0.007
	Sig.	0.383	0.94
	N	127	127
Reactive Hyperaemia Peak Perfusion (PU)	Correlation Coefficient	-0.013	-0.09
	Sig.	0.901	0.397
	N	90	91
Pulse wave velocity (m/s)	Correlation Coefficient	0.256	0.176
	Sig.	0.006	0.058
	N	116	117
Mean Common Carotid IMT Right (mm)	Correlation Coefficient	0.276	0.114
	Sig.	**0.002**	0.2
	N	127	127
Mean Bulb IMT Right (mm)	Correlation Coefficient	0.249	-0.096
	Sig.	0.014	0.349
	N	97	97
Mean Common Carotid IMT Left (mm)	Correlation Coefficient	0.15	-0.02
	Sig.	0.092	0.823
	N	127	127
Mean Bulb IMT Left (mm)	Correlation Coefficient	0.304	0.06
	Sig.	**0.002**	0.549
	N	103	103
Mean Peak SNP Response (PU)	Correlation Coefficient	-0.167	-0.091
	Sig.	0.085	0.349
	N	107	108

Mean Peak ACh Response (PU)	Correlation Coefficient	-0.082	-0.031
	Sig.	0.397	0.746
	N	109	110

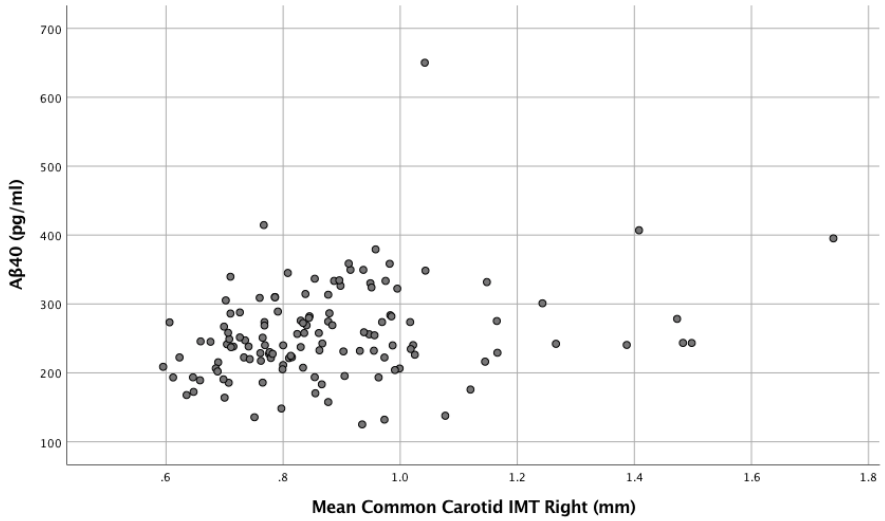

Figure 3-27: Scatter plot of β-amyloid 40 with right-sided mean common carotid IMT in Dundee subjects without T2DM, n=127, r=0.276.

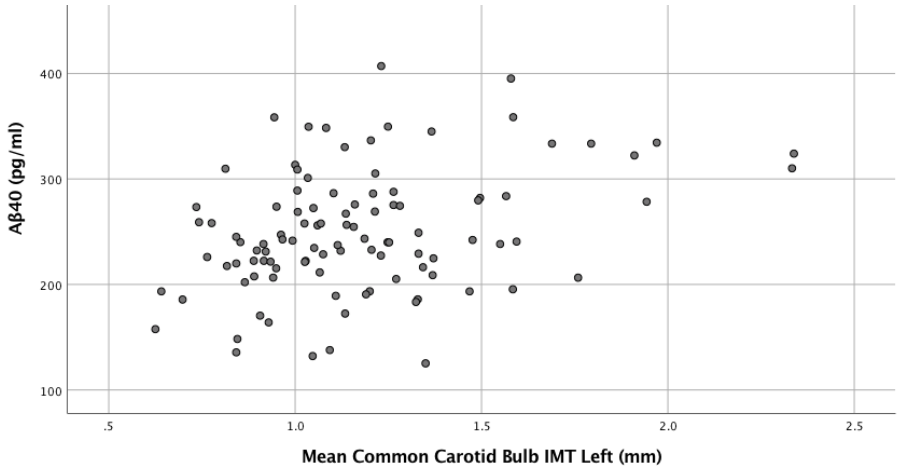

Figure 3-28: Scatter plot of β-amyloid 40 with left sided mean common carotid bulb IMT in Dundee subjects without T2DM, n=103, r=0.304.

Univariate correlations were then repeated after further subdividing Dundee subjects based on T2DM status. After the Bonferroni correction, significant univariate correlations were seen with β-amyloid 40 and mean right-sided CC and bulb IMT. No significant univariate correlations were seen with β-amyloid 42.

3.5.8.2 *Regression Models: Dundee Subjects Without T2DM*

Linear regression was then use to determine whether any associations with β-amyloid persisted after adjusting for common CVD risk factors. These linear regression models adjusted for established risk factors used in the Framingham and ASSIGN CVD risk scores. These were Age, Gender, SBP, HDL cholesterol and Total cholesterol. This model also adjusted for significant predictors of plasma β-amyloid in subjects without T2DM identified in chapter 1, in order to determine whether any effects of plasma β-amyloid were independent of these factors. When plasma β-amyloid 40 was included in the model, additional variables included were eGFR, age and height. When plasma β-amyloid 42 was included in the model, the additional factor included was eGFR. Each row with corresponding β-amyloid column in the table below represents an individual regression model.

Table 3-32: Summary of linear regression models with independent variables age, gender, systolic blood pressure, HDL cholesterol and total cholesterol with either β-amyloid 40 or 42 and eGFR, age, height or eGFR respectively.

Vascular Measurement – Dependent Variable	Aβ40 (pg/ml)		Aβ42 (pg/ml)	
	Beta	p-value	Beta	p-value
Reactive Hyperaemia Peak Perfusion (PU)	-0.033	0.787	-0.119	0.298
Reactive Hyperaemia Index (EndoPAT units)	0.057	0.588	-0.075	0.447
Pulse Wave Velocity (m/s)	0.125	0.161	0.048	0.563
Common Carotid IMT Right (mm)	0.107	0.262	0.024	0.789
Mean Bulb IMT Right (mm)	0.165	0.117	-0.036	0.723
Common Carotid IMT Left (mm)	0.104	0.307	-0.03	0.752
Mean Bulb IMT Left (mm)	**0.3**	**0.008**	0.099	0.359
Mean SNP Response (PU)	0.007	0.953	-0.039	0.717
Mean ACh Response (PU)	-0.051	0.661	-0.075	0.484

According to the linear regression models summarised above, the only significant and independent association of β-amyloid 40 in the Dundee non-T2DM cohort is with mean left-sided bulb IMT.

3.5.9 Analysis of Dundee Subjects With T2DM

The above analysis was then repeated in Dundee subjects with T2DM. The first step was to determine the association between plasma β-amyloid and biomarkers of vascular disease. Either Pearson or Spearman correlations were used, depending on the distribution of variables. The Bonferroni method was used to correct for multiple comparisons (new p value p=0.003). Scatterplots for significant correlations are displayed below.

3.5.9.1 *Univariate Correlations in Dundee Subjects with T2DM*

Table 3-33: Summary of univariate correlations of plasma β-amyloid with markers of vascular functional or structural change in Dundee subjects with T2DM.

Vascular Measurement	Correlation	Aβ40 (pg/ml)	Aβ42 (pg/ml)
Reactive Hyperaemia Index (EndoPAT) (units)	Correlation Coefficient	-0.063	-0.204
	Sig.	0.35	**0.002**
	N	220	220
Reactive Hyperaemia Peak Perfusion (PU)	Correlation Coefficient	-0.105	-0.35
	Sig.	0.215	**1.90E-05**
	N	142	142
Pulse wave velocity (m/s)	Correlation Coefficient	0.351	0.23
	Sig.	**2.00E-06**	**0.002**
	N	171	171
Mean Common Carotid IMT Right (mm)	Correlation Coefficient	0.139	0.108
	Sig.	0.039	0.112
	N	220	219
Mean Bulb IMT Right (mm)	Correlation Coefficient	0.127	0.004
	Sig.	0.117	0.963
	N	153	153
Mean Common Carotid IMT Left (mm)	Correlation Coefficient	0.082	-0.027
	Sig.	0.218	0.692
	N	225	225
Mean Bulb IMT Left (mm)	Correlation Coefficient	0.046	-0.008
	Sig.	0.54	0.919
	N	180	181
Mean Peak SNP Response (PU)	Correlation Coefficient	-0.13	-0.031
	Sig.	0.076	0.677
	N	187	186
Mean Peak ACh Response (PU)	Correlation Coefficient	-0.078	-0.067
	Sig.	0.288	0.361

	N	187	186

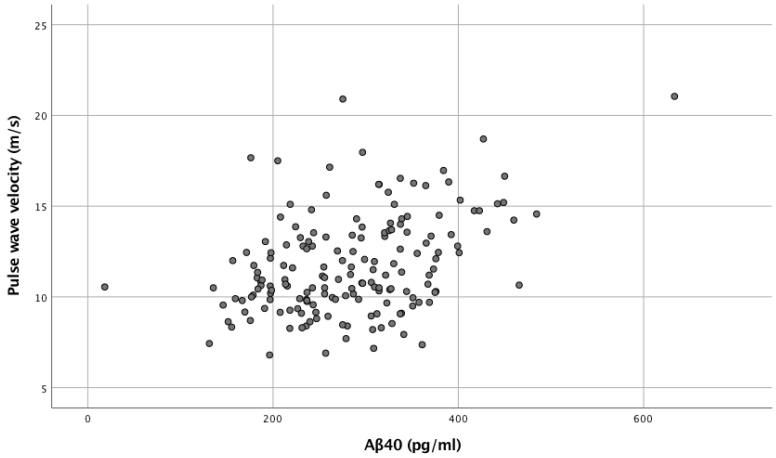

Figure 3-29: Scatter plot of β-amyloid 40 with pulse wave velocity in Dundee subjects with T2DM, n=171, r=0.351.

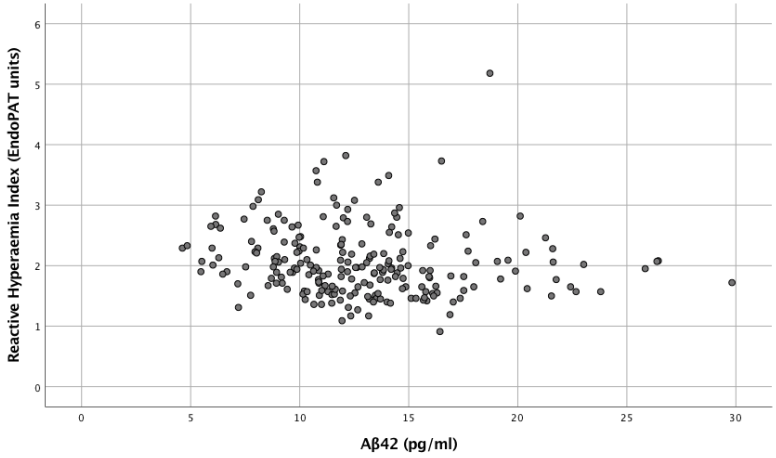

Figure 3-30: Scatter plot of β-amyloid 42 with reactive hyperaemia (EndoPAT) in Dundee subjects with T2DM, n=220, r=-0.204.

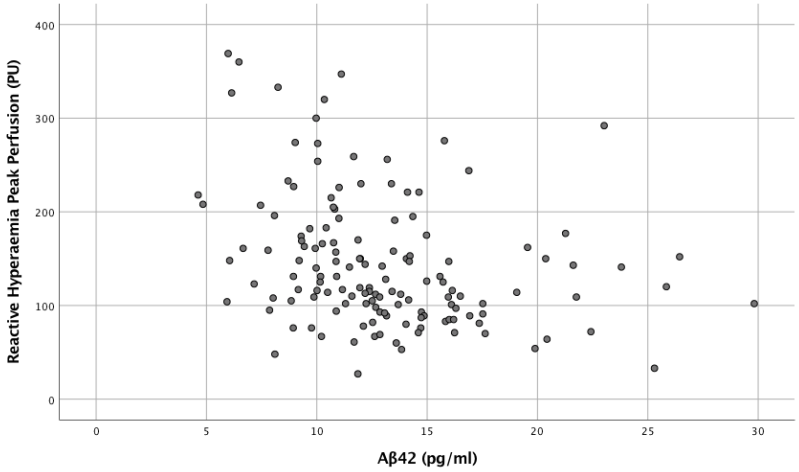

Figure 3-31: Scatter plot of β-amyloid 42 with peak reactive hyperaemia perfusion as measured by laser doppler, in Dundee subjects with T2DM n= 142, r=-0.350.

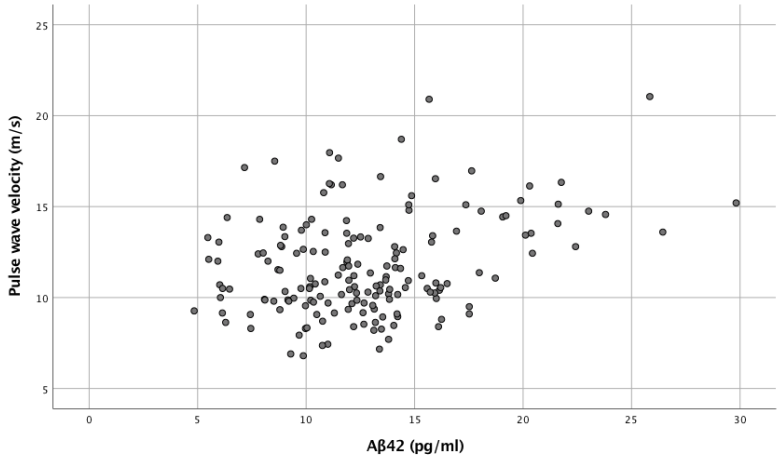

Figure 3-32: Scatter plot of β-amyloid 42 with pulse wave velocity in Dundee subjects with T2DM, n=171, r=0.230

The above univariate analyses show that after Bonferroni correction, a significant positive correlation is seen between β-amyloid 40 and pulse wave velocity in Dundee subjects with T2DM. With regards to β-amyloid 42, significant negative correlations can be seen with peak reactive hyperaemia response to temporary blood flow occlusion as well as RHI as measured by EndoPAT. As with β-amyloid 40, a significant positive correlation is seen with β-amyloid 42 and pulse wave velocity.

3.5.9.2 *Linear Regression Models: Dundee subjects with T2DM*

As before, the next step was to determine whether any associations of plasma β-amyloid persists even after adjusting for conventional CVD risk factors as well as clinical determinants of plasma β-amyloid identified in the previous chapter. The following linear regression models adjusted for age, gender, total cholesterol, HDL cholesterol and peripheral systolic blood pressure and either β-amyloid 40 or 42. Where models adjusted for β-amyloid 40, eGFR, insulin and diuretic use was also adjusted for. Where models adjusted for β-amyloid 42, eGFR and insulin use was also adjusted for. Each row in the table below represents an independent regression model. Beta refers to the standardised correlation coefficient.

Table 3-34: Linear regression models with independent variables age, gender, total cholesterol, HDL cholesterol and peripheral systolic blood pressure and either β-amyloid 40 with eGFR, diuretic use and insulin use or β-amyloid 42 and eGFR and insulin use.

Vascular Measurement – Dependent Variable	Aβ40 (pg/ml)		Aβ42 (pg/ml)	
	Beta	p-value	Beta	p-value
Reactive Hyperaemia Peak Perfusion (PU)	-0.052	0.619	**-0.235**	**0.021**
Reactive Hyperaemia Index (EndoPAT units)	-0.022	0.772	-0.09	0.228
Pulse Wave Velocity (m/s)	**0.242**	**0.002**	0.178	0.02
Common Carotid IMT Right (mm)	0.099	0.211	-0.043	0.577
Mean Bulb IMT Right (mm)	0.082	0.386	-0.028	0.762
Common Carotid IMT Left (mm)	0.06	0.454	-0.138	0.073
Mean Bulb IMT Left (mm)	0.016	0.859	-0.014	0.87
Mean SNP Response (PU)	-0.036	0.689	0.076	0.376
Mean ACh Response (PU)	-0.059	0.5	0.074	0.383

The above summary of numerous linear regression models shows that β-amyloid 40 is significantly and independently associated with increasing pulse wave velocity, and that β-amyloid 42 is significantly and independently associated with reduced reactive hyperaemia peak perfusion response, even after adjusting for conventional CVD risk factors and determinants of β-amyloid.

3.5.10 Analysis of the Exeter Baseline Cohort:

The analysis performed for the Dundee subgroup was then repeated for the Exeter subgroup.

3.5.10.1 *Univariate Correlations in the Exeter Baseline Cohort*

In order to begin exploring how plasma beta amyloid varies with different measures of structural and functional change in the Exeter baseline cohort, univariate analysis was performed using correlations. Depending on the distribution of parameters, either Pearson or Spearman correlations were used. The Bonferroni method (dividing standard p=<0.05 by number of comparisons made) was used to adjust for multiple comparisons at univariate level. Significant associations after adjustment (p<0.003) are displayed below.

Table 3-35: Summary of univariate correlations of plasma β-amyloid with markers of vascular functional and structural change in the Exeter Baseline cohort

Vascular Measurement	Correlation	Aβ40	Aβ42
Reactive Hyperaemia Index (EndoPAT)	Correlation Coefficient	0.075	0.051
	Sig.	0.228	0.415
	N	258	258
Reactive Hyperaemia Peak Perfusion (PU)	Correlation Coefficient	0.077	0.033
	Sig.	0.228	0.604
	N	248	248
Pulse wave velocity (m/s)	Correlation Coefficient	0.165	0.074
	Sig.	0.008	0.241
	N	253	253
Mean common carotid artery IMT Right	Correlation Coefficient	0.074	0.04
	Sig.	0.222	0.516
	N	272	272
Mean bulb IMT Right	Correlation Coefficient	-0.125	-0.075
	Sig.	0.048	0.239
	N	249	249
Mean common carotid artery IMT Left	Correlation Coefficient	0.061	0.015
	Sig.	0.318	0.812
	N	269	269
Mean bulb IMT Left	Correlation Coefficient	0.045	0.043
	Sig.	0.491	0.51
	N	240	240
Mean Peak SNP Response	Correlation Coefficient	-0.107	0.031
	Sig.	0.086	0.625
	N	259	259
Mean Peak ACh Response	Correlation Coefficient	-0.066	-0.03
	Sig.	0.292	0.629
	N	259	259

The above analysis shows that after Bonferroni correction, no significant correlations were seen in the baseline Exeter cohort for neither β-amyloid 40 nor β-amyloid 42.

3.5.10.2 *Linear regression in the Exeter baseline cohort*

As performed previously, a regression model was then built to determine whether any associations seen during univariate analysis persisted after adjusting for common CVD risk factors. This model adjusted for age, gender, diabetes status, total cholesterol, HDL cholesterol as well as factors identified as significant predictors of plasma β-amyloid in chapter 1. For β-amyloid 40 in the baseline cohort these were eGFR and diuretic use, and for β-amyloid 42 these were eGFR, diuretic use and LDL cholesterol. Each row represents a separate linear regression model with the dependent variable highlighted. Beta refers to the standardised regression coefficient.

Table 3-36: Linear regression models with independent variables of age, gender, diabetes status, HDL cholesterol, total cholesterol, systolic blood pressure and either β-amyloid 40 with eGFR + diuretic use or β-amyloid 42 with eGFR + diuretic use + LDL cholesterol.

Vascular Measurement	Aβ40 (pg/ml)		Aβ42 (pg/ml)	
	Beta	p-value	Beta	p-value
Reactive Hyperaemia Peak Perfusion (PU)	0.077	0.259	-0.003	0.968
Reactive Hyperaemia Index (EndoPAT units)	0.042	0.513	0.005	0.942
Pulse Wave Velocity (m/s)	0.077	0.259	-0.027	0.606
Common Carotid IMT Right (mm)	-0.026	0.651	0.011	0.857
Mean Bulb IMT Right (mm)	-0.09	0.152	-0.008	0.906
Common Carotid IMT Left (mm)	-0.069	0.258	-0.036	0.563
Mean Bulb IMT Left (mm)	0.025	0.703	0.118	0.077
Mean SNP Response (PU)	-0.095	0.136	-0.01	0.874
Mean ACh Response (PU)	-0.024	0.711	-0.025	0.702

The above linear regression models showed that even after adjusting for conventional CVD risk factors and determinants of plasma β-amyloid, no significant independent associations were seen with any marker of functional or structural vascular integrity.

3.5.11 Analysis of Exeter Subjects Without T2DM

The same analysis was then repeated in Exeter subjects without T2DM. Depending on the distribution of variables, either the Mann-Whitney or independent T-tests were used. The Bonferroni method was used to correct for multiple comparisons, by dividing the p value 0.05 by number of comparisons made (adjusted p-value, p<0.003). Scatter plots for significant correlations are displayed below.

3.5.11.1 *Univariate Correlations in Exeter subjects without T2DM*

Table 3-37: Summary of univariate correlation of β-amyloid with markers of vascular functional or structural change in Exeter subjects without T2DM.

Variable	Correlation	Aβ40	Aβ42
Reactive Hyperaemia Index (EndoPAT units)	Correlation Coefficient	0.127	-0.084
	Sig.	0.192	0.385
	N	108	108
Reactive Hyperaemia Peak Perfusion (PU)	Correlation Coefficient	0.146	0.181
	Sig.	0.138	0.065
	N	105	105
Pulse wave velocity (m/s)	Correlation Coefficient	0.294	0.134
	Sig.	**0.002**	0.165
	N	109	109
Mean common carotid artery IMT Right (mm)	Correlation Coefficient	0.163	0.146
	Sig.	0.086	0.126
	N	111	111
Mean bulb IMT Right (mm)	Correlation Coefficient	-0.121	-0.159
	Sig.	0.224	0.11
	N	102	102
Mean common carotid artery IMT Left (mm)	Correlation Coefficient	0.069	0.067
	Sig.	0.474	0.484
	N	110	110
Mean bulb IMT Left (mm)	Correlation Coefficient	-0.071	-0.101
	Sig.	0.469	0.304
	N	105	105
Mean Peak SNP Response (PU)	Correlation Coefficient	0.004	0.129
	Sig.	0.97	0.191
	N	105	105
Mean Peak ACh Response (PU)	Correlation Coefficient	-0.017	-0.018
	Sig.	0.865	0.856

	N	105	105

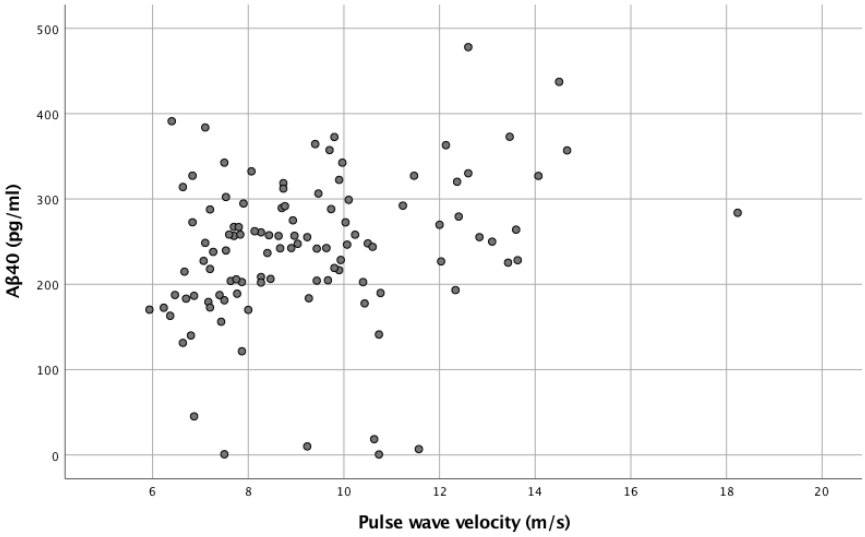

Figure 3-33: Scatter plot of β-amyloid 40 with pulse wave velocity in Exeter subjects without T2DM, n=109, r=0.294.

Univariate correlations were then performed in Exeter subjects without T2DM. After Bonferroni correction, the only significant association was a positive correlation between β-amyloid 40 and pulse wave velocity.

3.5.11.2 *Linear Regression Models: Exeter subjects without T2DM*

To determine whether any significant independent associations were seen after adjusting for common CVD risk factors, the following linear regression models were performed. These linear regression models adjusted for established risk factors used in the Framingham and ASSIGN CVD risk scores: Age, Gender, SBP, HDL cholesterol and Total cholesterol. This model also adjusted for significant predictors of plasma β-amyloid in subjects without T2DM identified in chapter 1, in order to determine whether any effects of plasma β-amyloid were independent of these factors. When plasma β-amyloid 40 was included in the model, additional variables included were eGFR, age and height. When plasma β-amyloid 42 was included in the model, the additional factor included was eGFR. Each row with corresponding β-amyloid column in the table below represents an individual regression model.

Table 3-38: Linear regression models with independent variables age, gender, systolic blood pressure, HDL cholesterol, total cholesterol and either β-amyloid 40 with eGFR + age + height or β-amyloid 42 with eGFR in Exeter subjects without T2DM.

Vascular Measurement	Aβ40 (pg/ml)		Aβ42 (pg/ml)	
	Beta	p-value	Beta	p-value
Reactive Hyperaemia Peak Perfusion (PU)	0.082	0.451	-0.14	0.19
Reactive Hyperaemia Index (EndoPAT units)	0.085	0.411	0.16	0.148
Pulse Wave Velocity (m/s)	0.147	0.054	0.132	0.089
Common Carotid IMT Right (mm)	0.036	0.692	0.153	0.099
Mean Bulb IMT Right (mm)	-0.158	0.122	-0.189	0.078
Common Carotid IMT Left (mm)	-0.094	0.331	-0.01	0.918
Mean Bulb IMT Left (mm)	-0.043	0.66	-0.064	0.533
Mean SNP Response (PU)	0.04	0.71	0.176	0.107
Mean ACh Response (PU)	0.095	0.379	0.104	0.341

After adjusting for conventional CVD risk factors as well as determinants of β-amyloid levels, no significant independent associations were seen in Exeter subjects without T2DM.

3.5.12 Analysis of Exeter Subjects With T2DM

Next, the Exeter subjects with T2DM were examined. The first step was to determine the association between plasma β-amyloid and biomarkers of vascular structural or functional change. Either Pearson or Spearman correlations were used, depending on the distribution of variables. The Bonferroni method was used to correct for multiple comparisons (new p value $p<0.003$). Scatterplots for significant correlations are displayed below.

3.5.12.1 *Univariate Correlations in Exeter subjects with T2DM*

3-39: Summary of univariate correlations of plasma β-amyloid with markers of vascular functional or structural change in Exeter subjects with T2DM.

Variable	Correlation	Aβ40	Aβ42
Reactive Hyperaemia Index (EndoPAT units)	Correlation Coefficient	0.054	0.129
	Sig.	0.515	0.115
	N	150	150
Reactive Hyperaemia Peak Perfusion (PU)	Correlation Coefficient	0.031	-0.08
	Sig.	0.713	0.34
	N	143	143
Pulse wave velocity (m/s)	Correlation Coefficient	0.109	0.056
	Sig.	0.192	0.502
	N	144	144
Mean CC IMT Right (mm)	Correlation Coefficient	0.022	-0.021
	Sig.	0.786	0.792
	N	161	161
Mean CC Bulb IMT Right (mm)	Correlation Coefficient	-0.124	-0.022
	Sig.	0.133	0.793
	N	147	147
Mean CC IMT Left (mm)	Correlation Coefficient	0.062	-0.012
	Sig.	0.435	0.878
	N	159	159
Mean CC Bulb IMT Left (mm)	Correlation Coefficient	0.121	0.13
	Sig.	0.163	0.134
	N	135	135
Mean Peak SNP Response (PU)	Correlation Coefficient	-0.229	-0.055
	Sig.	0.004	0.495
	N	154	154
Mean Peak ACh Response (PU)	Correlation Coefficient	-0.155	-0.086
	Sig.	0.055	0.289
	N	154	154

Again, after Bonferroni correction, no significant correlations were seen for neither β-amyloid 40 nor β-amyloid 42.

3.5.12.2 *Linear Regression Models: Exeter Subjects with T2DM.*

As before, the next step was to determine whether any associations of plasma β-amyloid persists even after adjusting for conventional CVD risk factors as well as clinical determinants of plasma β-amyloid identified in the previous chapter. The following linear regression models adjusted for age, gender, total cholesterol, HDL cholesterol and peripheral systolic blood pressure and either β-amyloid 40 or 42. Where models adjusted for β-amyloid 40, eGFR, insulin and diuretic use was also adjusted for. Where models adjusted for β-amyloid 42, eGFR and insulin use was also adjusted for. Each row with corresponding β-amyloid column in the table below represents an independent regression model.

Table 3-40: Linear regression models with independent variables age, gender, systolic blood pressure, HDL cholesterol, total cholesterol and either β-amyloid 40 with eGFR + insulin + diuretic use or β-amyloid 42 with eGFR + insulin use in Exeter subjects with T2DM.

Vascular Measurement	Aβ40 (pg/ml)		Aβ42 (pg/ml)	
	Beta	p-value	Beta	p-value
Reactive Hyperaemia Peak Perfusion (PU)	0.057	0.539	-0.098	0.29
Reactive Hyperaemia Index (EndoPAT units)	0.02	0.819	0.05	0.569
Pulse Wave Velocity (m/s)	-0.017	0.807	-0.075	0.285
Common Carotid IMT Right (mm)	-0.079	0.327	-0.065	0.411
Mean Bulb IMT Right (mm)	-0.036	0.669	0.079	0.34
Common Carotid IMT Left (mm)	-0.073	0.376	-0.057	0.477
Mean Bulb IMT Left (mm)	0.077	0.388	**0.178**	**0.045**
Mean SNP Response (PU)	**-0.178**	**0.04**	-0.073	0.396
Mean ACh Response (PU)	-0.109	0.206	-0.078	0.36

Interestingly, after adjusting for conventional CVD risk factors as well as clinical determinants of β-amyloid 40 and 42, significant independent associations were seen with β-amyloid 40 and mean peak SNP response as well as β-amyloid 42 with mean left-sided bulb IMT.

3.5.13 Summary of Key Findings

- **SUMMIT Baseline cohort**
    - β-amyloid 40 is significantly and independently associated with increased peak reactive hyperaemia response and pulse wave velocity
    - β-amyloid 40 is also significantly and independently associated with reduced response to SNP and ACh iontophoresis
    - β-amyloid 42 was significantly and independently associated with a reduced reactive hyperaemia response
    - At enrolment into the study, higher β-amyloid 40 levels were associated with increased number of LEAD cases
    - No associations were seen with follow up adverse cardiovascular outcomes or overall mortality

- **SUMMIT Non-T2DM cohort**
    - β-amyloid 40 is significantly and independently associated with increased pulse wave velocity
    - β-amyloid 42 levels were significantly associated with increased peak response to SNP iontophoresis

- **SUMMIT T2DM cohort**
    - β-amyloid 40 is significantly and independently associated with increased peak reactive hyperaemia response and pulse wave velocity
    - β-amyloid 40 is also significantly and independently associated with reduced response to SNP and ACh iontophoresis
    - No significant independent associations were seen with β-amyloid 42
    - At enrolment into the study higher β-amyloid 40 levels were associated with a higher number of LEAD cases

- **Dundee Baseline Cohort**
    - The Dundee and Exeter cohorts were significantly different across a number of different baseline clinical parameters

- β-amyloid 40 is significantly and independently associated with increased pulse wave velocity
- β-amyloid 42 was significantly and independently associated with a reduced reactive hyperaemia response

- **Dundee Non-T2DM Cohort**
  - β-amyloid 40 is significantly associated with increased left-sided bulb IMT

- **Dundee T2DM Cohort**
  - β-amyloid 42 is significantly and independently associated with increased pulse wave velocity

- **Exeter Baseline Cohort**
  - No significant independent associations were seen

- **Exeter Non-T2DM Cohort**
  - No significant independent associations were seen

- **Exeter T2DM Cohort**
  - β-amyloid 40 is also significantly and independently associated with reduced response to SNP iontophoresis

3.6 Discussion

β-amyloid has previously been extensively investigated primarily in the context of AD. However, in recent years a number of studies have shown that it may play a role in CVD. The present study looked at the association of plasma β-amyloid 40 and 42 with markers of functional and structural vascular change as well as with cardiovascular outcomes over a follow up period of 4-6 years. To date, this is the first comprehensive study associating both β-amyloid 40 and 42 with a multitude of vascular structural and functional assessments. Additionally, it is the first study to date to look at these relationships in subjects with and without diabetes separately.

3.6.1 Findings from the SUMMIT Cohort – β-amyloid and Arterial Stiffness

Several studies have previously reported a strong association between β-amyloid 40 and arterial stiffness (38,120,121). These findings were reproduced in the present study, where this association persisted even after adjusting for established CVD risk factors as well as clinical determinants of plasma β-amyloid levels identified in chapter 2. This held true in the SUMMIT baseline cohort, as well as subjects with and without T2DM when looked at individually. Therefore, these findings reinforce those previously published in the literature. In addition, by controlling for determinants of plasma β-amyloid levels, this study suggests that a direct relationship could exist between β-amyloid and pulse wave velocity, and that these findings are not merely as a result of an association between other factors such as renal function. However, the exact mechanism of how β-amyloid could contribute to arterial stiffness is still unknown.

In general, increasing arterial stiffness is considered to be a paradigm of vascular ageing and is thought to occur primarily as a result of changes to the extracellular matrix (ECM). These changes include increased collagen deposition, and reduced elastin concentrations (122). However, whether β-amyloid contributes to the process via the same set of mechanisms is unclear. A possible explanation for how β-

amyloid could alter arterial stiffness relates to the extensive research done in the context of CAA. CAA is a poorly understood disease entity characterised by β-amyloid deposition in small to medium sized blood vessels of the brain. It manifests clinically as recurrent, often debilitating intracerebral haemorrhages and associated haemorrhagic stroke syndromes. Pathologically, it results in stenosis of the vessel lumen, fibrinoid necrosis and microaneurysm formation (123). In the context of this pathological state, β-amyloid has been shown to be toxic to VSMCs, and to result in changes to the ECM component of the cerebral vasculature. In fact, the steps by which β-amyloid results in changes to vascular structure have been well documented. In the first stages, β-amyloid is found to accumulate at the basal lamina (124,125). As CAA progresses, β-amyloid deposition extends beyond the basal lamina into VSMC layers. Once it has reached this layer, VSMC death occurs in a dose dependent manner and can result in a complete lack of a functional response to vasoactive stimuli (126). With yet more advanced stages of CAA, the vascular media is completely replaced by β-amyloid deposits and lacks any surviving VSMCs. Even at these later stages, the endothelial cells remain functional, but are seen to have abnormal morphologies (127).

In terms of β-amyloid's effects on the ECM, in vitro studies have shown that β-amyloid can induce MMP-9 activity (127). Interestingly, MMP-9 has been associated with systemic arterial stiffness in a range of different studies. Yasmin et al. report that increased levels of MMP-9 and increased elastase activity is associated with increasing arterial stiffness in both healthy individuals and subjects with isolated systolic hypertension (128). The same group has also shown that single nucleotide polymorphisms (SNPs) in the MMP-9 gene significantly affect arterial stiffness in healthy individuals (129). Therefore, the ability of β-amyloid to induce MMP-9 levels could be a potential explanation for the strong association between increased plasma β-amyloid 40 levels and increasing arterial stiffness. Unfortunately, MMP-9 was not among the numerous MMPs measured in the SUMMIT database. In the future, measuring MMP-9 in these samples could be of interest in an attempt to determine the relationship between β-amyloid, MMP-9 and arterial stiffness.

3.6.2 β-amyloid and the Vascular Response to Acetylcholine and Sodium Nitroprusside

In light of the effects of β-amyloid on VSMCs described above, the associations between plasma β-amyloid and laser Doppler imaging with iontophoresis data must be discussed in the context of microvascular function. The present study is the first to investigate the association between plasma β-amyloid and vascular responsiveness to ACh and SNP assessed by means of laser Doppler imaging and iontophoresis. When looking at univariate correlations, a significant negative correlation between plasma β-amyloid 40 levels and peak perfusion response to SNP and ACh exists. More importantly, these significant associations persist in the SUMMIT baseline and SUMMIT T2DM cohorts even after adjusting for conventional CVD risk factors and significant clinical determinants of plasma β-amyloid 40 levels. Several important points need to be discussed as a result of these observations. Firstly, a significant association between plasma β-amyloid 40 and peak SNP or ACh was not seen in SUMMIT subjects without T2DM. It is possible, that this relationship is specific to subjects with T2DM, and that the analysis of the SUMMIT baseline cohort was skewed by the comparatively larger number of subjects with T2DM. However, this is unlikely, as the regression model in the SUMMIT baseline cohort adjusted for diabetes status. Therefore, the more likely explanation for this observation is that the cohort of SUMMIT subjects without T2DM was the smallest and possibly underpowered to demonstrate any associations. Indeed, even strong associations seen in the baseline cohort and T2DM cohort disappeared in the analysis looking at Exeter and Dundee subgroups separately, which was understandably associated with a large reduction in the number of subjects. Another important point that needs to be discussed is the significant association with both peak ACh and peak SNP responses. This could be interpreted in different ways. Firstly, it is possible that increased β-amyloid 40 is associated with reductions both endothelium dependent and endothelium independent vasodilator responses. However, it is also possible that increased β-amyloid 40 is only associated with endothelium independent pathways (SNP response), but because the response to ACh targets steps proximal in the pathway, the response to ACh is also diminished. Literature findings related to this matter provide little explanation as they are largely

conflicting. As discussed in the previous section, β-amyloid has been shown to cause VSMC death (37), which would understandably result in a diminished response to SNP administrations. However, numerous in vitro studies have shown that β-amyloid exerts vasoactive properties in an endothelium dependent manner. Indeed, findings from studies in mice by our group would also suggest that β-amyloid exerts its effects in an endothelium dependent manner. This leads on to another interesting point for discussion, as in the preceeding mouse study by our research group, β-amyloid 42 as opposed to 40 was found to be the main culprit. In the present study, while β-amyloid 42 was significantly associated with increased peak response to SNP in one regression model (SUMMIT subjects without T2DM), this was by no means a consistent observation and is possibly due to chance. Nevertheless, findings from our preceeding animal study are in direct contrast with current findings. It is possible that these discrepancies are due to inter-species variation.

3.6.3 β-amyloid and Reactive Hyperaemia

Further adding to the dilemma surrounding the association of plasma β-amyloid with markers of functional change in the vasculature are the present findings of a positive association between reactive hyperaemia and β-amyloid 40 and while a significant negative association between β-amyloid 42 and reactive hyperaemia in the SUMMIT baseline cohort. A significant positive association was also seen between β-amyloid 40 and reactive hyperaemia in the SUMMIT T2DM cohort. This would suggest that higher plasma β-amyloid 40 levels are associated with better reactive hyperaemia responses to temporary blood flow occlusion. This seems to contrast other results in this study, as similarly to the vascular response to ACh, reactive hyperaemia is a response mediated primarily in an endothelium-dependent manner. However, it is well recognised that the reactive hyperaemia response occurs via mechanisms different to those involved in the vasodilator response to ACh. It is thought, that during temporary blood flow occlusion, vasodilator metabolites such as adenosine accumulate due to the state of tissue hypoxia. These cause downstream vasodilation. As the blood flow is restored, down stream vasodilation causes reduced

resistance to blood flow, which in turn increases the shear stress on upstream vessels (130). However, how β-amyloid impacts on this process and why differing effects are seen when assessing different forms of endothelium dependent response is unclear.

3.6.4 β-amyloid and cIMT

To date, no studies have looked directly at the association between β-amyloid and cIMT thickness. In the main analysis of the present study, significant associations between plasma β-amyloid 40 levels and right sided cIMT existed, but only when performing univariate analyses. After adjusting for conventional risk factors and clinical determinants of β-amyloid, β-amyloid 40 was only significantly associated with increasing cIMT in the Dundee Non-T2DM cohort. Therefore, it is possible that any associations within the univariate analysis were due to chance.

3.6.5 β-amyloid and Cardiovascular Outcomes

One previous study has reported that after adjusting for age, gender, eGFR, CRP, Troponin T and left ventricular ejection fraction, plasma β-amyloid 40 independently predicted cardiovascular death as well as major adverse cardiac events in patients with a history of coronary heart disease (38). In the present study, neither plasma β-amyloid 40 nor 42 were found to be significant predictors of any cardiovascular events or overall mortality. While it is possible that β-amyloid is not of prognostic value when it comes to CVD risk prediction, a likely explanation is that this study was underpowered to demonstrate any associations. Indeed, the study by Stametelopoulos et al. recruited almost double the number of subjects included in this database (38).

3.6.6 Effect of diabetes status on associations of β-amyloid with markers of vascular and structural and functional health

In the present study, a number of subgroup analyses were performed with subjects divided based on diabetes status. An interesting and consistent observation throughout the analyses of the SUMMIT and Exeter cohorts was that β-amyloid was significantly and independently associated with a larger number of structural and functional markers of vascular health in the T2DM subgroups when compared to the non-T2DM subgroups. However, in analyses of the SUMMIT and Dundee cohorts, the grouped baseline analysis (both T2DM and Non-T2DM subjects) revealed the largest number of significant independent associations. Therefore, a likely explanation for the stronger and more numerous associations seen in T2DM subgroups compared to non-T2DM subgroups, is the number of subjects included in individual analyses and therefore statistical power. Therefore, it is unlikely that β-amyloid associates uniquely with vascular markers in T2DM. This is an important observation, as the animal study this project was based on suggested that β-amyloid may be a contributor to vascular dysfunction in a manner unique to T2DM and diet-induced obesity models.

3.6.7 Limitations of Study:

There are several potential limitations of this study that must be considered. Firstly, although analysis of the grouped cohort revealed some interesting associations of plasma β-amyloid with markers of vascular structural and functional health, the subgroup analysis by centre only partially reproduced these observations. The most likely explanation for this is that the sub-group analysis by centre was associated with too large a reduction in number of subjects.

Another observation that must be addressed, is the small group of subjects in the Exeter cohort with extremely low levels of both β-amyloid 40 and 42 (see scatter plots from the Exeter sub-group analysis). Although these are seemingly outliers, they were included in any analysis due to the inability to determine the reason behind such observations. The levels of β-amyloid measured in this group was still well within the minimum detection limit for the assay used. Additionally, these samples were scattered across a number of assay plates, suggesting that is unlikely to be due to an individual assay failure. As β-amyloid levels were only measured as a one-off measurement due to the limited amount of plasma available and the need to

also measure other circulating biomarkers, it was impossible to exclude a qualitative problem with the sample. When looking at the characteristics of this small subgroup, there was no single factor that would link these subjects together. Across the group a range of different ages, BMIs, blood pressures and medications can be observed. In the future, it would be interesting to determine the genotype of these individuals. Given that this seemingly outlying group of subjects could not be excluded from the cohort and was present only in the subjects from Exeter, it is possible that it skewed some of our results and contributed to some of the conflicting results observed between the two cohorts. To resolve this, an analysis excluding subjects with extremely low levels of β-amyloid could be carried out and used as a reference point to determine if outliers account for some of the differences between centres.

Although we lack knowledge regarding the source of β-amyloid, the findings in this study perhaps help shed light on the potential elimination mechanisms. In fact, one of the strongest associations uncovered in Chapter 2 was the strong association of eGFR and other related markers of renal function with plasma β-amyloid in both diabetic and non-diabetic populations. Should β-amyloid play a direct role in the development of vascular disease it is possible that renal elimination could be of therapeutic significance not only for this disease entity, but also for other β-amyloid associated pathological states including AD or cerebral amyloid angiopathy. This would, however, only hold true under the assumption that plasma β-amyloid levels are at an equilibrium with cerebral β-amyloid levels, and that a transport mechanism exists between the two.

The strong association between β-amyloid and eGFR however, brings with it a large set of limitations for plasma β-amyloid as a potential biomarker. Although pathological processes affecting the kidneys and resulting in lower eGFR are plentiful, the commonest cause of end stage renal failure in the developed world is diabetic nephropathy (131). Therefore, it is debatable whether β-amyloid would be a useful biomarker of CVD in patients with diabetes. Although it is possible that β-amyloid could be a useful predictor of cardiovascular disease in earlier stages of diabetes, another complicating matter is the observation that patients in the early stages of type 2 diabetes experience renal hyperfiltration as a result of osmotic

diuresis (132). Indeed, it is now well accepted in a number of different pathophysiological models of diabetic nephropathy that hyperfiltration is the first stage of the pathological process (132). However, if β-amyloid is shown to be a direct contributor to the pathological process of CVD as opposed to being only a coincident biomarker, any effects of eGFR on plasma β-amyloid levels could become less important.

This directly relates to another possible limitation of β-amyloid as a biomarker given its strong association with renal function. Renal dysfunction itself is a very strong risk factor for cardiovascular disease, in fact, in patients with chronic kidney disease, cardiovascular disease is one the commonest cause of death (133). Therefore, it is possible that β-amyloid could simply be acting as a biomarker of renal function, with poor renal function being the underlying causal association.

When looking at results from the outcome analysis, several limitations can be discussed. Firstly, although plasma β-amyloid was not shown to be a significant predictor of cardiovascular outcomes at end of follow up, on most occasions neither were classical risk factors such as total cholesterol, HDL cholesterol, diabetes status, age or systolic blood pressure also. This could be due to a number of different reasons, the most likely being that there were very small amounts of outcomes accumulated over the course of the follow up period. In total, there were 18 deaths, 17 diagnoses of claudication, 12 transient ischaemic attacks, 6 strokes, 20 diagnoses of unstable angina and 14 cases of acute myocardial infarction. When considered as individual entities, the numbers in each category are relatively low and as such can result in a significantly underpowered analysis. However, when grouping all outcomes into a composite dependent variable, conventional risk factors for cardiovascular disease still remained statistically insignificant. Additionally, although a subgroup analysis dividing subjects based on diabetes status was performed for all other analyses, little value was thought to be gained from doing so in outcome analysis, as these would underpower statistical tests even further. Ideally, follow up would continue for a longer period of time to allow for the accumulation of more cardiovascular events.

4 Summary

β-amyloid is a peptide molecule best known for its role in the development of AD. Surprisingly, despite an evidence base and research funding far bigger than most other diseases, a large number of unknowns continue to surround β-amyloid. Perhaps unusually, this study investigated β-amyloid in the context of a different disease process, CVD. The background to this study stems from currently unpublished findings from previous animal studies carried out by our research group. Briefly, plasma β-amyloid was shown to be increased in mice with diet induced obesity, and increased plasma β-amyloid 42 levels by means of β-amyloid 42 infusions were associated with reduced vascular responsiveness to ACh and SNP. Lowering β-amyloid, by pharmacologically inhibiting the enzyme responsible for mediating its rate limiting step, was shown to restore vascular responsiveness.

In light of these findings, this thesis therefore aimed to translate these findings into the human population. More specifically, this thesis aimed to investigate the association of β-amyloid with markers of vascular structural and functional change as well as with clinically manifest CVD. Additionally, in an attempt to shed some light on the processes that dictate β-amyloid kinetics in the circulation, this project also investigated the clinical determinants of plasma β-amyloid. These objectives were fulfilled by statistically analysing the pre-existing SUMMIT database in combination with retrospective measurement of plasma β-amyloid by the Immunoassay Biomarker Core Laboratory situated in Ninewells Hospital, Dundee.

The first analysis in this study (chapter 2) looked at the clinical determinants of plasma β-amyloid in our SUMMIT cohort. Although limited information on associations of β-amyloid with determinants such as eGFR previously existed, there is no information regarding how well these findings translate to other populations such as younger subjects or subjects without dementia. Although a relatively limited analysis, this process helped highlight the strength of the relationship between eGFR and plasma β-amyloid, but also unveiled some new interesting associations including a relationship between insulin use and diuretic use with plasma β-amyloid levels.

Not only did this analysis enable us to then adjust for these variables in models looking at β-amyloid in the context of CVD, but will also hopefully be of value in the field of AD, where plasma β-amyloid has been a much sought after biomarker, but also one that to date remains conflicting and elusive.

The second analysis aimed to translate findings from previous animal studies into the human population. An analysis of the SUMMIT cohort confirmed previous reports of an independent association of β-amyloid 40 with arterial stiffness as measured by pulse wave velocity. Additionally, β-amyloid 40 was also shown to be independently associated with impaired peak response to SNP and ACh in the SUMMIT baseline and T2DM cohorts, as assessed by means of laser Doppler iontophoresis. Less consistent findings in this analysis suggest that a possible independent association may exist between β-amyloid 40, cIMT and reactive hyperaemia as well as β-amyloid 42 and reactive hyperaemia. These associations may be of interest in future research. Lastly, in the present study, β-amyloid 40 or 42 were not found to be independently associated with CVD outcomes in our cohort over a follow-up period of 4-6 years. However, this analysis was likely underpowered and therefore reliable conclusions cannot be drawn solely on the basis of our data. Again, this area may be worth revisiting in the future, with more robust databases and longer follow up periods.

Overall, this translational study supports some of the findings previously reported by our group as well as other research groups. Although a number of unanswered questions remain, and the potential predictive value of β-amyloid has not been unveiled, it is clear that β-amyloid has affinity for the vasculature and is associated with markers of vascular and structural change.

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